The Efficacy of Suspended Broodstock Cages as a Restoration Strategy for the European Flat Oyster *Ostrea edulis* Linnaeus, 1758: A Case Study in the Solent, UK.

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The thesis is submitted in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy of the University of Portsmouth

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Abstract

Faculty of Science and Health, School of Biological Sciences

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The efficacy of suspended broodstock cages as a restoration strategy for the European flat oyster *Ostrea edulis* Linnaeus, 1758: A case study in the Solent, UK.

By

Luke David Helmer

The European flat oyster *Ostrea edulis* has been subject to multiple stressors that have resulted in the functional extinction of many populations, those that remain are fragmented and require active intervention to accommodate their recovery. One of the last self-sustaining populations in Europe was, until recently, present within the Solent, but extraction pressure, disease and sporadic recruitment resulted in its recent collapse. This study aimed to determine the abundance of the remaining oyster populations in relation to the invasive American slipper limpet *Crepidula fornicata*, suspected to occupy the ecological niche made available by the removal of *O. edulis*. Oyster populations were minimal within the three harbours surveyed, < 0.2 ±0.2 individuals / m^2 (Portsmouth, Langstone and Chichester), whereas, *C. fornicata* was extremely abundant, present in densities of up to 4043 ±2374 individuals / m^2. In an attempt to increase reproduction and larval output, therefore seabed recruitment, mature oysters were purchased from the local fishery and placed into broodstock cages at high stocking densities across the Solent. Monitored across two years, the effect of stocking density, pressure-washing and environmental conditions on mortality, larval production and disease prevalence were observed. Mortality was seasonal peaking after an increase in brooding activity and spawning. Brooding occurred within the expected temporal period, peaking in June, and larval brood sizes were significantly different between oysters in different marinas, as well as between full- and half-density populations, despite there being no significant difference in the proportion of brooding adults. Disease status was monitored and geographical variation in *Bonamia ostreae* prevalence was observed. The detection of *Bonamia exitiosa* in the region increases the described range of the parasitic species. With design and operational modifications, the broodstock cage system can be used as an effective management tool to assist with increasing initial densities of larvae in recruitment limited areas in the early stages of restoration.

Declaration of Authorship and Word Count

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

Word Count: 44,088

Luke David Helmer

30/09/2019

"*I love oysters. It's like kissing the sea on the lips*"

Léon-Paul Fargue (1876-1947)

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Contents

List of Tables

List of Figures

List of Abbreviations

BA: Sampling location in Portsmouth Harbour previously Ben Ainslie Racing

BLAST: Basic Local Alignment Search Tool

bp: Base pairs

CEFAS: Centre for Environment, Fisheries and Aquaculture Science

CI: Condition index

coxI gene: Cytochrome c oxidase subunit I gene

DNA: Deoxyribonucleic acid

EA: Environment Agency

EDTA: Ethylenediaminetetraacetic acid

FHI: Fish Health Inspectorate

ftu: Formazin turbidity unit

GLM: General linear model

GP: Gosport Marina - sampling location

GVA: Gross value added

HP: Hamble Point Marina -sampling location

HY: Hythe Marina Village - sampling location

IMS: Institute of Marine Sciences

INNS: Invasive non-native species

ITS: Internal transcribed spacer

Kb: Kilobase

MC: Mercury Marina - sampling location

MEGA X: Molecular evolutionary genetics analysis: version X

MLS: Minimum landing size

MSD: Maximum shell depth

MSL: Maximum shell length

MSW: Maximum shell width

NOSAP: Native Oyster Species Action Plan

NT: Northney Marina - sampling location

NVZ: Nitrate vulnerable zone

DO: Dissolved oxygen

OV: Ocean Village Marina - sampling location

PAHs: Polycyclic aromatic hydrocarbons

PCBs: Polychlorinated biphenyls

PCO: Principle coordinates analysis

PCR: Polymerase chain reaction

PES: Polyethersulfone

PH: Port Hamble Marina - sampling location

PRIMER: Plymouth Routines in Multivariate Ecological Research package

psu: Practical salinity units

rDNA: Ribosomal DNA

RNA: Ribonucleic acid

RV: Research vessel

SD: Standard deviation

SE: Standard error

SORP: Solent Oyster Restoration Project

IFCA: Inshore Fisheries & Conservation Authority

SP: Sparkes Marina - sampling location

SS: Southsea Marina - sampling location

SST: Sea surface temperature

SSU: Small subunit

SW: Saxon Wharf - sampling location

TAE: Tris-acetate-EDTA

TBT: Tributyltin

UK: United Kingdom

UKBAP: UK Biodiversity Action Plan

UP: University of Portsmouth Research platform - sampling location

UV: Ultraviolet

WFD: Water Framework Directive

Chapter 1

General Introduction

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1.1. The biology of *Ostrea edulis* (Linnaeus, 1758)

The European flat oyster *Ostrea edulis* is a sessile bivalve mollusc belonging to the Ostreidae family of true oysters and is known by a variety of common names throughout Europe, including the term "*Pied de cheval*" ("horse hoof"), used by the French to describe extremely large, old oysters. The typical life span of *O. edulis* is estimated to be between five and ten years, however, larger individuals have been reported to have lived 18 - 24 years (Roberts *et al.*, 2010). Natural mortality has been observed to occur within 10 - 25 % of populations (Orton, 1923).

The genus *Ostrea*, within the Ostreidae, includes numerous extant species that are closely related (Fig. 1.1) with similarities in morphology, biology and reproduction. The majority of the available literature focuses on the commercially exploited species, including *O. angasi* (G. B. Sowerby II, 1871), *O. lurida* (Carpenter, 1864), *O. chilensis* (Küster, 1844), as well as *O. stentina* (Payraudeau, 1826) and *O. equestris* (Say, 1834). Other species include *O. puelchana* (d'Orbigny, 1842), *O. conchaphila* (Carpenter, 1857), *O. denselamellosa* (Lischke, 1869), *O. angelica* (Rochebrune, 1895) and *O. megodon* (Hanley, 1846).

Ostrea edulis is similar in external appearance to many other *Ostrea* species, especially *O. angasi*, but is distinctive in relation to *Crassostrea* spp., including the widely commercially grown Pacific oyster *Crassostrea gigas* that is present across much of Europe. While external morphology can be variable, *O. edulis* is often relatively oval in shape and consists of a flat dorsal valve that sits within the concave ventral valve, joined at the hinge. The dorsal valve is predominantly brown with blue iridescent banding, whilst the ventral valve exhibits phenotypic polymorphisms that include brown, red, green, white, yellow, pink, purple and blue colourations. These valves are usually comprised of three layers, the first being the periostracum; a thin outer layer which sits on the middle section or prismatic layer of calcite. The innermost layer, normally pearly white in colour, is formed from

aragonite (Walne, 1974). The similarity in external morphology has caused confusion with cryptic members of this genus, with the example of *O. lurida* and *O. conchaphila* on the Pacific coast of North America (Polson *et al.*, 2009; Raith *et al.*, 2015).

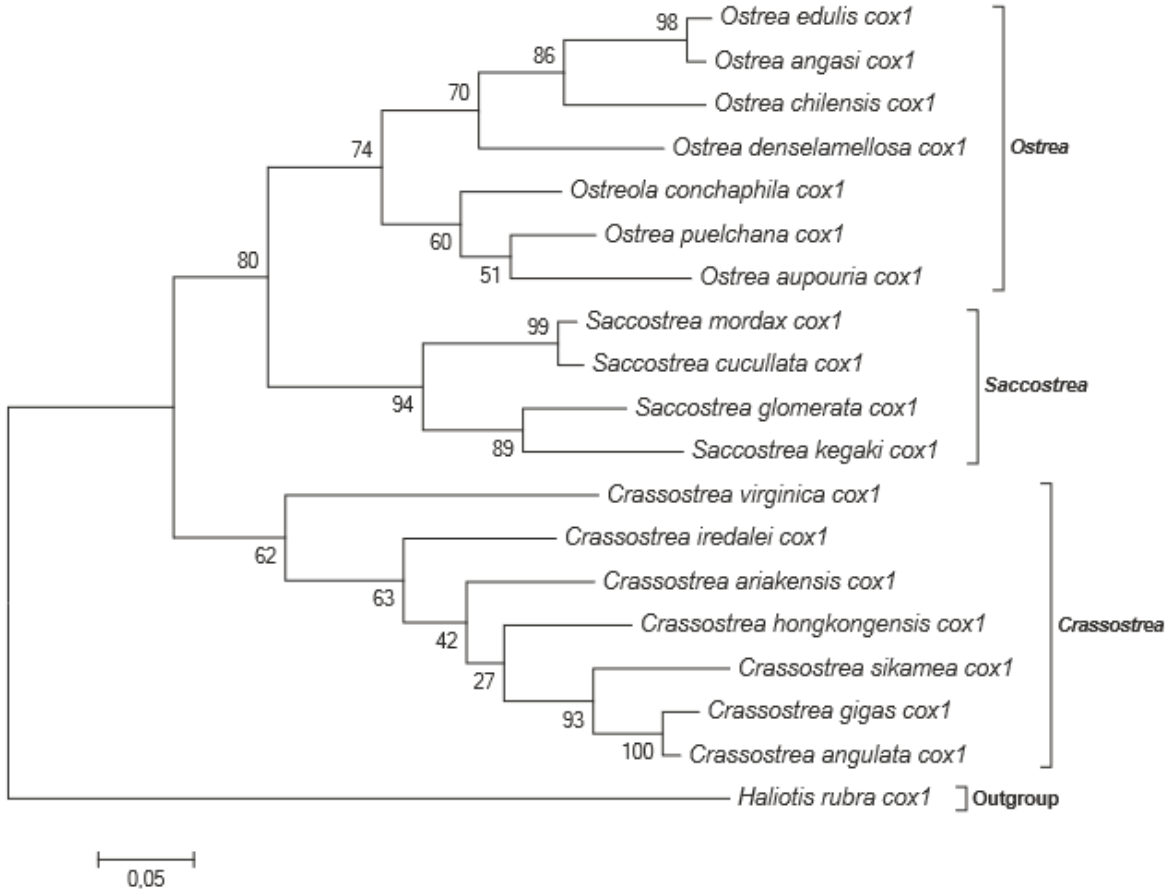


Figure 1.1. Phylogenetic tree based on *cox1* from all published *Ostrea*, *Saccostrea* and *Carassosrea* spp. at the time of publication, 2011. *Ostreola conchaphila* is now *Ostrea conchaphila*. Source: Danic-Tchaleu *et al.* (2011).

Internal anatomy of *O. edulis* consists of multiple organs, including a single adductor muscle that is firmly attached to both valves and that is separated into two sections. The quick muscle is translucent and allows the oyster to close swiftly when the presence of a threat or disturbance is detected, whilst the catch muscle (opaque) is required to keep the valves closed for extended periods of time when conditions are not favourable. The elasticity of the ligament allows for the controlled opening of the valves, in a gaping position, for feeding to take place (Walne, 1974). Flow of water into the mantle cavity is controlled by

the movement of the largest fold of the mantle on opposite sides of the cavity whilst the two other folds are involved in secretion of the shell and further cementation (Harper, 1992). Particles of potential food material within incoming water, entering via the inhalant chamber, are moved along the gill filaments and sorted. Filtered water is passed to the exhalant chamber and extruded from the oyster whilst the extracted food and other particles move towards the mouth.

The particles small enough to have been retained, and often joined together by mucus, are then further sorted by the labial palps which further extrude larger particles, not suitable for consumption, into the mantle cavity. This discarded material, termed pseudofaeces, accumulates throughout the feeding process and is regularly extruded by the vigorous closure of the two valves, this is distinguished by its formation as fluffy piles. In contrast, true faeces are extruded via the anus as firm ribbons and is the waste product of food particles that pass through the complex digestive tract, comprised of the oesophagus, stomach, mid gut, digestive diverticula and rectum (Fig. 1.2).

Other internal structures include the gonad, which, in good condition forms a 2 - 3 mm thick layer and indicates the individual is in prime reproductive state, often described as being “ripe”. There is no obvious dimorphism between the gonads of the two sexes, due to the hermaphroditic nature of the species, however the incidence of sperm or eggs provides an indication to the current state of a particular individual. Reproductive processes and larval development are discussed further in section 1.2. The heart (A and V Fig. 1.2) is located anteriorly of the adductor muscle and completes the basic anatomy of *O. edulis*, see Yonge (1926) and Walne (1974).

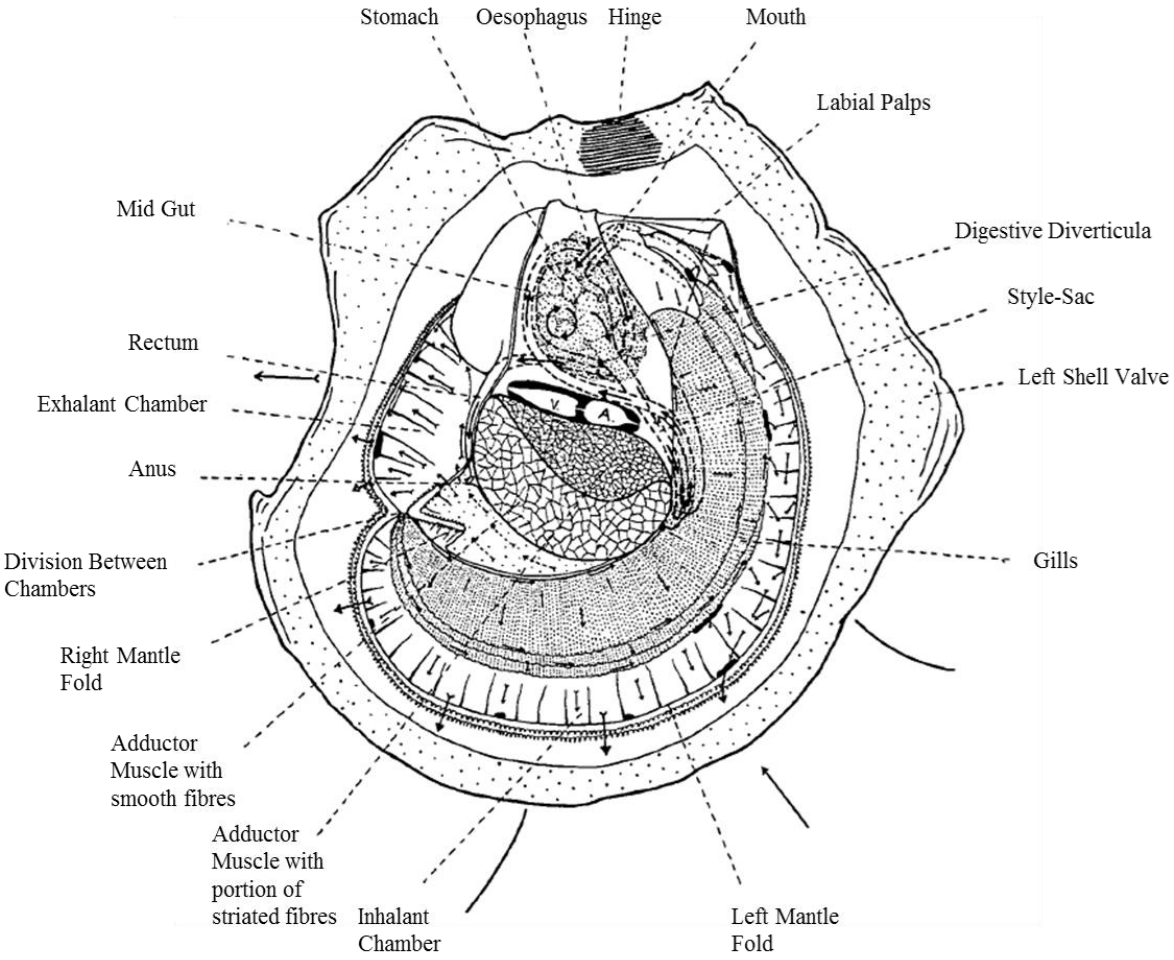


Figure 1.2. The internal anatomy of *Ostrea edulis* contained within the ventral (left) valve, modified from Yonge (1926). Large external arrows indicate the movement of water in and out of the oyster, plain and feathered internal arrows indicate movement of ingoing and outgoing water movement, respectively. Broken arrows, except in the gut, indicate currents under surfaces.

1.2. Reproduction and larval development

Members of the genus *Ostrea* are known for their sporadic and inconsistent recruitment which can, in part, be explained by their reproductive cycle. As protandrous hermaphrodites the juveniles of *O. edulis* normally develop as males until they are approximately two years of age, after which alternation between genders occurs (Orton, 1922; Walne 1974). Sperm released by individuals functioning as males, is taken up by individuals functioning as females, via the inhalant siphon, where it fertilises the awaiting eggs. The sperm is initially released as spermatozeugmata and held within an extracellular matrix around a core of acellular vesicles that are denser than seawater (Foighil, 1989). This mechanism allows the sperm to remain demersally distributed within the water column, increasing the likelihood of successful entry into the female mantle cavity.

Subsequent development leads to the spherical embryo developing a crown of motile cilia and ectodermal invagination as they enter the trochophore stage (Waller, 1981). Followed by the evagination of the shell gland, secretion and enlargement of the shell, and formation of internal structures within the shell to reach the veliger "D" larvae stage at approximately 140 - 190 µm in diameter (Acarli and Lok, 2009). The mass within the mother oyster gradually darkens with three notable stages of development being white, grey and black "sick" (McGonigle *et al.*, 2016). Upon reaching the black sick stage the larvae are prepared for release. This brooding period lasts for between one - two weeks for *O. edulis* and *O. lurida* but is prolonged within *O. chilensis* as the larvae of this species continue their development within the mantle cavity to almost direct development (Toro and Chaparro, 1990; Chaparro *et al.*, 1993). The number of larvae produced by *O. edulis* has been reported to be proportional to the size (mean diameter), less so age, of the adult brooding them (Walne, 1974) (Fig. 1.3) and is substantially more than that of a marketable sized (50 mm) *O. lurida*, 250,000 - 300,000 (Hopkins, 1937), again brood size relates to adult shell length.

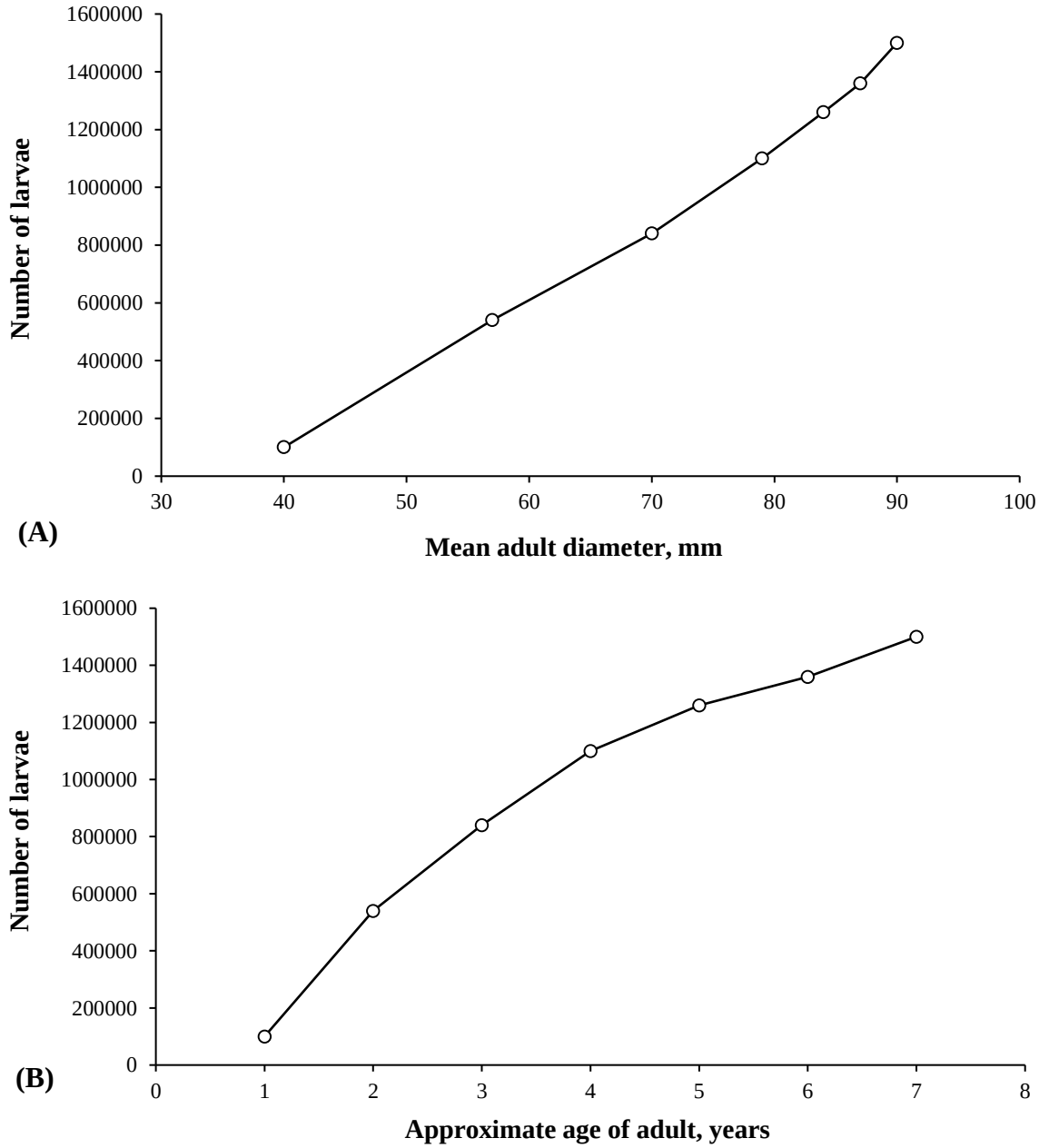


Figure 1.3. Number of larvae produced by *Ostrea edulis* in relation to (A) mean adult diameter (mm) and (B) approximate adult age (years) according to Walne (1974).

Newly released larvae are equipped with one ciliary band that contains long cilia used for locomotion within the water column, as development continues into the pediveliger stage, approximately 240 - 300 µm, three ciliary bands become distinguishable (Erdmann, 1935). As the pediveliger approaches the metamorphosis stage a ciliated foot develops, this occurs medially via an outgrowth of the body wall ectoderm (Erdmann, 1935). The larval

behaviour also changes as the larvae use this foot to survey for suitable substrata to settle upon. Many oyster species, including *O. edulis*, display gregarious behaviour, showing preferential settlement on adult individuals of the same species (Bayne, 1969; Rodriguez-Perez *et al*., 2019).

If a suitable substratum is not detected the larvae are able to relocate and reassess another area, a process that can be repeated multiple times until the larvae chance upon an appropriate substratum (Walne, 1974). The byssal gland at the base of the foot then secretes a drop of cement as the larvae rocks back and forth, once this has occurred the ventral valve of the larvae is applied to the cement. Secretion of a periostracum from the mantle also assists with the cementation and occurs continuously throughout the process (Harper, 1992). This cementation is a permanent process, and, unlike the byssus threads of mussels, the oyster cannot reapply attachment mechanisms in this manner (Cole, 1938; Hickman and Gruffydd, 1971; Cranfield, 1973). Following permanent cementation, the larvae are known as spat, with the velum, foot, anterior adductor mussel and eye spot all disappearing (Waller, 1981). Gills form initially as single filaments that connect over time and elaboration continues to resemble that of the adults, at the same time the adductor muscle moves from a posterior position to a more central position. After 3 - 4 days have passed, the spat resembles the body plan of the adults and growth continues into adult form (Fig. 1.4).

Sexual maturity, thus the capability to produce offspring, usually occurs between 2 - 3 years after settlement (Orton, 1937). Brooding of significantly reduced quantities of larvae, in comparison to older, larger oysters, has however been observed during the first year, post settlement (Gerbe, 1876; Lacaze-Duthiers, 1893; Dantan, 1913 in Cole, 1941; Orton, 1922).

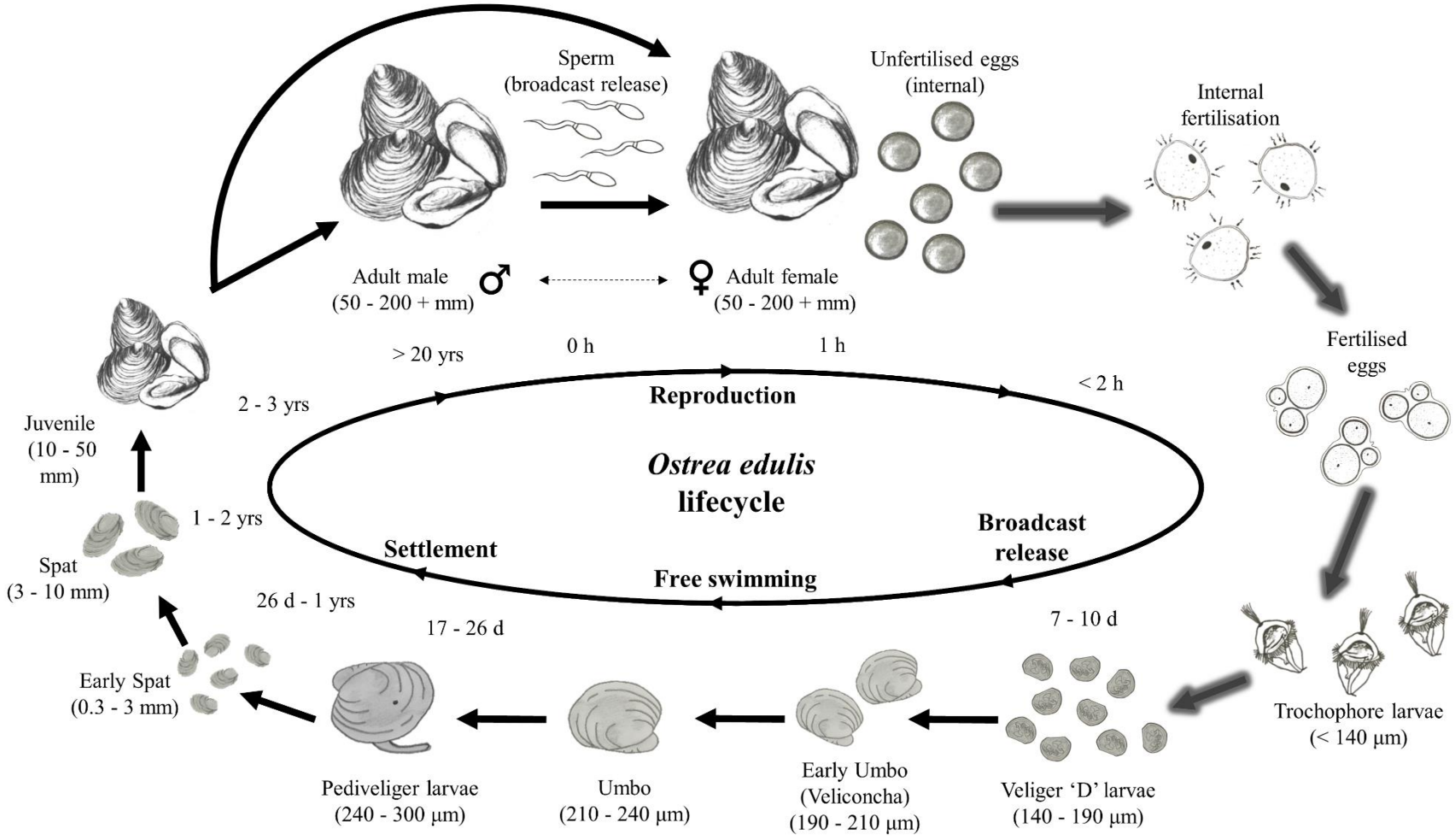


Figure 1.4. Lifecycle of *Ostrea edulis*. Arrows with glow effect indicate stages that occur internally within the female oyster pallial (mantle) cavity, plain arrows indicate stages that occur externally. Approximate sizes and timings are based upon information from Hu *et al*. (1993), Acarli & Lok (2009), Food and Agriculture Organization of the United Nations (FAO) (2016) and, Loosanoff, *et al*. (1966), Pascual (1972) and Tanaka (1981), cited within Hu *et al*. (1993). Images of life stages are not to scale.

1.3. Ecological and economic importance of *Ostrea edulis*

The term "ecosystem engineer" is used to describe any organism that directly or indirectly modulates the availability of resources to other species, by causing physical state changes in biotic or abiotic materials. *Ostrea edulis*, along with other oyster species, fits within the classification of an autogenic engineer, whereby the physical structure provided whilst alive and by the remaining shells when deceased changes the environment (Jones *et al*., 1994).

The vast majority of research regarding oyster related ecosystem services has been focused around *Crassostrea* species, with particular attention paid to the intertidal vertical reef forming eastern oyster *C. virginica* (Gutiérrez *et al.*, 2003; Coen *et al*., 2007; Coen & Grizzle, 2007, see Table 1.1). Although ecological services such as habitat creation, water filtration, bento-pelagic coupling and carbon / nitrogen sequestration are likely to be provided by *Ostrea* species, including *O. edulis*, they are yet to be quantified in detail. This combination of services provided by oyster reefs not only provides ecological enhancements but has significant economic value.

The services provided by these systems (excluding harvest) is economically valued between $5,500 - $99,000 USD per hectare, per year (Grabowski *et al.*, 2012). The potential gross value added (GVA) relating to *O. edulis* in the Solent, under different water quality scenarios using direct GVA from harvesting and indirect GVA from supply-chain expenditure, has been estimated to be between £301,221 - £306,266, using data from 2015 - 17 (Williams and Davies, 2018). The role of aquaculture in providing these services has also been investigated (Gentry *et al.*, 2019), again a knowledge gap exists for *Ostrea* species.

Simultaneously to the provision of services provided by existing and restored oyster populations, the loss of oyster reefs is associated with loss of these ecological and economic services (Thurstan *et al.*, 2013; Zu Ermgassen *et al.*, 2013) and detrimental impacts on the

resulting habitat and marine ecosystem (Lenihan and Peterson, 1998). Restoration costs can be recovered in a period of 2 - 14 years when no harvesting takes place; however, the cost of restoration is not recovered when destructive harvesting takes place (Grabowski *et al.*, 2012), again this is *Crassostrea* specific.

Table 1.1. A brief summary of the known ecosystem services provided by oysters and oyster reefs, obtained with a literature search using a combination of key words including "Oyster", "*Ostrea edulis*" and "Ecosystem Service(s)".

Service	Species	Oysters or oyster reefs	Reference(s)
Water/seston filtration	*O. edulis*	Oysters	Walne, 1974
	C. virginica	Oysters	Officer *et al.*, 1982; Newell, 2004; Ehrich & Harris, 2015
Biogeochemical processes	*C. virginica*	Oyster reefs	Chambers *et al.*, 2018
Nekton biomass	*C. virginica*	Oyster reef	Peterson *et al.*, 2003; Stunz *et al.*, 2010; Humphries and Peyre, 2015
Carbon sequestration	*C. virginica*	Oyster reefs	Fodrie *et al.*, 2018
Nitrogen removal	*C. virginica*	Oyster reefs	Kellogg *et al.*, 2013; Smyth *et al.*, 2015
Habitat creation:	*O. edulis*	Oysters	Smyth and Roberts, 2010;
Habitat use			Zwerschke *et al.*, 2016
Faunal abundance	*O. puelchana*	Oysters	Romero *et al.*, 2013a, b
and diversity	*C. virginica*	Oyster reefs	Wells, 1961; Bahr and
Refugia			Lanier, 1981; Zimmerman
Nesting			*et al.*, 1989; Coen, 1999;
			Harding and Mann, 2001;
			Gutiérrez *et al.*, 2003;
			Grabowski, 2004;
			Grabowski *et al.*, 2005;
			Tolley and Volety, 2005;
	C. rivularis	Oyster reefs	Karp *et al.*, 2018
	S. glomerata	Oyster reefs	Quan *et al.*, 2009
	S. cucullata	Oysters	McAfee *et al.*, 2016, McAfee and Bishop, 2019
			Raghukumar *et al.*, 1991

Table 1.1 continued

Service	Species	Oysters or oyster reefs	Reference(s)
Historic associated biota	*O. alvarezii*	Oysters	Romero *et al.*, 2018
Benthic-pelagic coupling	*C. virginica*	Oysters	Smyth *et al.*, 2013
Physical-biological coupling	*C. virginica*	Oyster reefs	Lenihan, 1999
Shoreline protection	*C. virginica*	Oyster reefs	Scyphers et al., 2011; La Peyre et al., 2015
Benefits to other habitats	*C. virginica*	Oyster reefs	Newell and Koch, 2004
Abiotic stress amelioration	*S. glomerata* *S. cucullata*	Oyster reefs Oyster reefs	McAfee *et al.*, 2016, 2017 McAfee *et al.*, 2018
Multiple	*C. virginica* Multiple	Oyster reefs Ecosystems	Grabowski and Peterson, 2007; Baggett *et al.*, 2014, zu Ermgassen *et al.*, 2016 Gillies *et al.*, 2018

1.4. *Ostrea edulis* history, distribution and exploitation

Ostrea edulis first appears in the fossil record during the Miocene (15.97 million years ago) and has subsequently been found in records across the continent as well as in Egypt (Wood, 1848; Jiménez & Braga, 1993; Fariñas-Franco *et al.* 2018; Fossilworks, 2019). Currently *O. edulis* is typically observed inhabiting coastal and estuarine environments, which range from the intertidal down to 80 meters in depth, within a salinity range of 18 - 40 ‰ (Jackson, 2007). Populations would have once covered extensive areas of European waters that would have equated to over 25,000 km ^{2} (Olsen, 1883, Fig. 1.5) and would have occurred in the form of dense aggregations in bed and reef structures. These, once abundant, populations provided a source of sustenance for human populations for centuries, with the earliest shell midden records dating back to the Mesolithic period (Gutiérrez-Zugasti *et al*., 2011).

Although native to waters across Europe, stretching from Morocco to Norway and incorporating the Mediterranean and Black Seas (Zaitsev and Alenxandov, 1998; Gosling, 2003 based on Carriker and Gaffney, 1996), anthropogenic translocations have extended the biogeographical range of *O. edulis* into North America (FAO, 2006; Trimble *et al.*, 2009), Canada (Newkirk, 1989; Bataller *et al*., 2006; Burke *et al*., 2008), Mexico (Funes and Jiménez, 1989), South Africa (FAO, 2007 - 11, Haupt *et al*., 2010), Japan (Fujiya, 1970), Australia (Morton *et al*., 2003; Molnar *et al*., 2008), Mauritius and Pacific islands (Bromley *et al.*, 2016a).

Large scale cultivation and management of the species extends back to the Roman Empire (Günther, 1897) and the continued large-scale extraction throughout the industrial revolution is highlighted by the 120,000 strong fleet of oyster dredgers that, in 1864, supplied 700 million oysters to London alone (Philpots, 1890). The 80 million oysters harvested annually in the Bay of Biscay, prior to 1859, were valued at £10,000 (Sullivan, 1870),

equivalent to £1.2 million today. The long-standing impression that the oceans provided inexhaustible source of fish and shellfish can be seen elsewhere, including the historic shell piles that have been observed to contain 5 x 10^{12} shells in France (Gruet & Prigent, 1986 in Goulletquer & Heral, 1997).

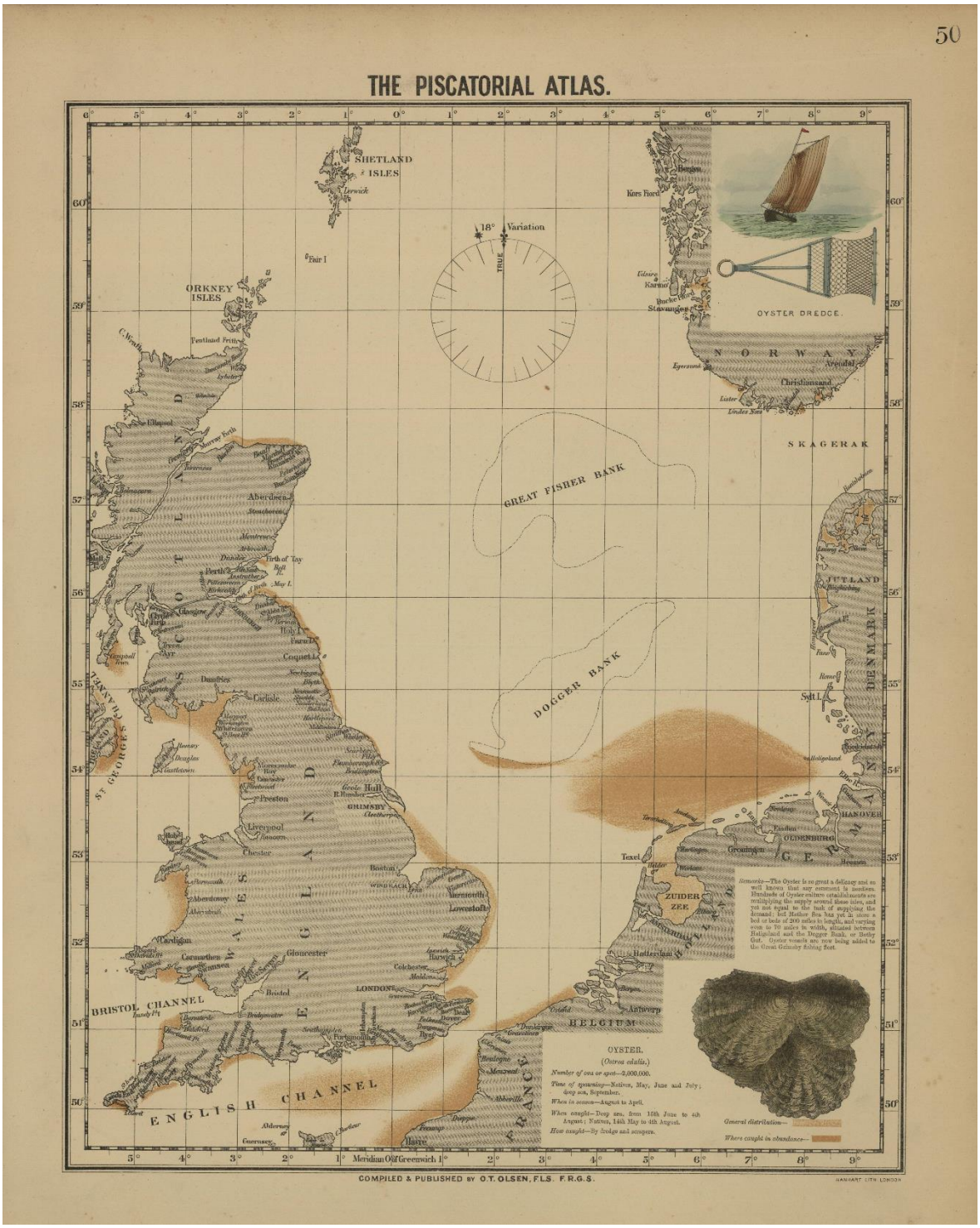


Figure 1.5. The Piscatorial Atlas of Olsen (1883) portraying the known distribution of *Ostrea edulis* (orange) around the coast of the UK, the English Channel and the North Sea.

However, only a year after the landing of 700 million oysters, reports were published stating that the bed around the UK had nearly been exhausted and that there was the need to erect artificial breeding grounds (South of England Oyster Company, 1865). The notion that oyster fisheries may indeed be exhaustible was declared in the inaugural address of Thomas Henry Huxley at the opening of the Fisheries Exhibition in 1882 (Fig. 1.6):

"Thus I arrive at the conclusion–first that oyster fisheries may be exhaustible; and, secondly, that for those which lie outside the territorial limit no real protection is practically possible. In the case of the oyster fisheries which lie inside the territorial limit the case is different. Here the State can grant a property in the beds to corporations or to individuals whose interest it will become to protect them efficiently. And this I think is the only method by which fisheries can be preserved."

The idea that other fisheries were not subject to such exhaustion highlights that there was still a lack of awareness of the consequences of long-term large-scale anthropogenic fishing activities:

"I believe, then, that the cod fishery, the herring fishery, the pilchard fishery, the mackerel fishery, and probably all the great sea fisheries, are inexhaustible; that is to say, that nothing we do seriously affects the number of the fish. And any attempt to regulate these fisheries seems consequently, from the nature of the case, to be useless."

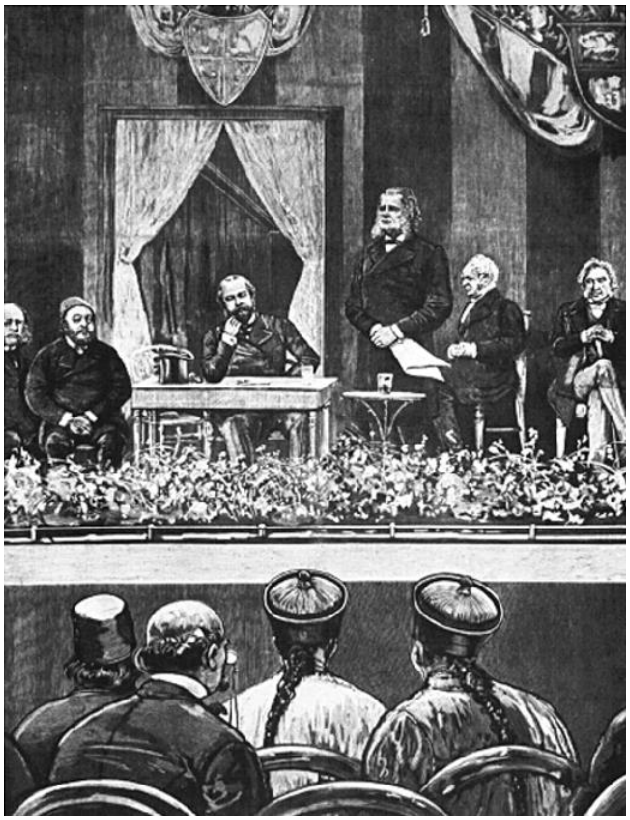


Figure 1.6. Thomas Henry Huxley's inaugural address to the Fisheries Exhibition in 1882 where he first mentions the exhaustibility of oyster populations.

Shortly after this address there are accounts of oyster beds within the Moray Firth, previously of good quality oysters, being fished to extinction by Colchester oystermen (Anon, 1882; Young, 1888).

This story of excessive exploitation is, unfortunately, much the same for almost all other commercially exploited oyster species, especially members of the genus *Ostrea*, across the globe. Many populations in coastal bays and harbours have been shown to suffer a reduction of 99 %, to the point where those populations are considered to be functionally extinct. It is estimated that a total of 85 % of populations have been lost globally, making oyster habitats one of the most imperilled habitats of any (Beck *et al*., 2009, 2011).

Commercial fishing of *Ostrea angasi* in Australia took place for over one hundred years from the beginning of the colony in 1836, with the peak harvest and collapse of populations occurring in the mid to late 19th century. Initial legislation was introduced in 1853, with the addition of minimum landing sizes, a closed season, closed areas and penalties in 1873 (Governor, 1873 in Alleway & Connell, 2015). A licencing system and exclusive rights to newly discovered beds were also introduced in 1885 (Inspector of Oyster Fisheries, 1886; Kirby, 2004). These acts reiterated that the over extraction of the species was understood but was not substantial enough to prevent collapse. At present, the last known *O. angasi* reef exists in Georges Bay, Tasmania (Gillies *et al.*, 2018). The associated destruction of the benthic habitat is also likely to have indirectly prevented successful settlement on other species such as *Ecklonia radiata* which has been shown to facilitate the rapid colonisation, > 5 kg live oysters / m^2, of *O. angasi* (Shelamoff *et al.*, 2019).

The Olympia oyster *Ostrea lurida* became commercially extinct on the Pacific coast of North America prior to 1930 due to overexploitation during the late 19th century. During this period, from the mid-1860s to 1907, the removal of over one hundred million oysters per annum and total estimates of four billion individuals and seventy-seven million

kilograms of shell are reported (Swan, 1857; Collins, 1892; Townsend, 1896; Kincaid, 1986). As with *O. edulis* in Europe and *O. angasi* in Australia, the overexploitation was recognised in 1855 and the fishery was closed thereafter from June to August (Steele, 1957), however, this was not enough to prevent the populations collapsing. The lack of stock recovery has been linked to the extraction of dense subtidal native oyster shell accumulations, as well as competition from other introduced oyster species and fouling organisms (Trimble *et al.*, 2009).

Ostrea chilensis has also been subject to commercial dredging for a century and a half, since 1867, in New Zealand (Cranfield *et al.*, 1999). Annual harvest was estimated to be 1 - 2 million individuals from Port William and Halfmoon Bay beds (Hunter, 1906), with a reduction in catch rates, to below that of an economically viable fishery, occurring within four to five years (Pearson, 1877). This story is much the same for the beds across the Foveaux Strait as they were discovered and harvested, with the introduction of steam-powered vessels in 1913, further increasing landings which were in-turn doubled by the introduction of diesel-powered machinery in 1936. Peak extraction occurred in 1967 when 127 million oysters were removed (Cranfield *et al.*, 1999).

Not all *Ostrea* species occur in great enough abundance to warrant their commercial exploitation, an example of this *Ostrea puelchana* which has not suffered the catastrophic losses, seen by other species, due to their occurrence in low densities across a limited geographic range (Castro & Le Pennec, 1988). Commercial cultivation of *O. puelchana* only began in 1995 and by 1997 the majority of the stock had perished due to the presence of a *Bonamia*-like parasite (Kroeck and Montes, 2005). However, it is evident that the historic quantities and frequency of extraction left many exploited *Ostrea* species with little chance of sustained natural recruitment, thus, the remaining populations are incapable of replenishing the immense numbers of broodstock lost to the fisheries.

1.5. Current restoration networks

The importance of restoring oysters is now well understood. The extreme demise of *O. edulis* across all of Europe requires urgent anthropogenic action to prevent the species from becoming functionally extinct. This urgency prompted the recent formation of UK and EU networks, which are utilising the available and invaluable information provided by the Oyster Habitat Restoration Monitoring and Assessment Handbook (Baggett *et al.*, 2014) to define the restoration requirements for *O. edulis*.

The University of Portsmouth and Zoological Society of London, funded by the John Ellerman Foundation, created the Native Oyster Network UK & Ireland (https://nativeoysternetwork.org/) to facilitate an ecologically coherent and collaborative approach to the restoration that is currently ongoing across several projects (Fig. 1.7). The Solent Oyster Restoration Project (SORP) is one of seven active restoration projects included within the network around the UK and Ireland and was established after the Blue Marine Foundation were contacted by the Southern Inshore Fisheries and Conservation Authority (IFCA), who recorded an acute decline in Solent oyster landings from 200 to 20 tonnes over a five year period (Southern IFCA, 2014 cited in Gravestock *et al.*, 2014). The SORP was formed to "*restore the status of the native oyster in Solent waters so that a healthy, self-sustaining population will improve ecosystem health, increase biodiversity and enhance water quality"*.

Another local initiative is the Chichester Harbour Oyster Partnership Initiative (CHOPI) whom established one of the first restoration efforts in the Solent area. This collaboration between the Sussex Inshore Fisheries and Conservation Authority (IFCA), Southern IFCA, Natural England, researches at the University of Southampton and CEFAS in Weymouth, Chichester Harbour Conservancy and fishermen, first attempted re-laying broodstock oysters in the Harbour at 40 / m^2 over three 30 m^2 areas (Eagling, 2012).

In a similar manner to the UK & Ireland network, the Native Oyster Restoration Alliance (NORA) (https://noraeurope.eu/), initiated by the German Federal Agency for Nature Conservation (BfN) and the Alfred Wegener Institute (AWI), was established to facilitate best practice on a Europe-wide scale (Pogoda *et al.*, 2019). The alliance now includes numerous projects across Europe in areas such as the Baltic Sea, North Sea, English Channel, French Atlantic coast and Adriatic Sea. The Global Shellfish Rehab Network has also begun compiling all known shellfish reef restoration project worldwide (https://shellfishrehab.net/).

Figure 1.7. Locations of current native oyster restoration projects (numbers) and fishery or production areas (oyster symbol) that have signed up to the Native Oyster Network UK & Ireland. Membership correct as of September 2019. See nativeoysternetwork.org for further details. Credit: Celine Gamble, network coordinator.

1.6. Barriers for *Ostrea edulis* restoration, with reference to the Solent

The numerous detrimental impacts facing practitioners attempting to restore *O. edulis* populations across Europe are summarised at the end of this section in Figure 1.11. Although overfishing was the principal reason for the decline, other influences contributed to, and accelerated, that decline with many of these factors the focus of restoration efforts.

1.6.1. Scarcity of remaining populations

By far the most substantial barrier to the restoration and recovery of *O. edulis* populations at present is the low standing stocks, a result of the chronic overfishing that has removed the source of almost all populations across Europe. This includes the larger "*mother oysters*" or "*Pied de cheval*" that would have produced the most substantial broods formerly allowing for the self-sustaining nature of the once colossal populations. Recent estimations indicate densities in the Solent are as low as 0.05 oysters / m^2 (Kamphausen, 2012) and that few areas across the Solent contain any viable populations (Southern IFCA, 2017, 2018b). The situation is arguably most severe in coastal areas along the German North Sea where the species is classed as extinct and must undergo a species reintroduction (Pogoda, 2019).

The removal of this ecosystem engineer had multiple knock on effects as successful brooding of larvae at high densities, and therefore settlement of substantial numbers of spat, requires high densities of mature individuals (≤ 1.5 m apart, Guy *et al.*, 2019). The broodstock themselves provide a suitable settlement substratum for larvae and if removed in a destructive manner, via dredging, then the benthic habitat that may have also provided settlement substratum becomes unsuitable (Lenihan & Peterson, 1998).

1.6.2. Limited and unreliable supply chain

The historic levels of overfishing have meant that many farmers and aquaculture specialists made the transition into production and cultivation of *Crassostrea gigas*, a species

that is easier to produce and on-grow in an intertidal environment, making it an obvious commercial choice. Within the UK there are currently eleven fisheries and / or production companies and seven restoration projects that have associated themselves with the 'UK Native Oyster Network UK & Ireland'. It is unlikely that the current supply will be able to meet the demand from restoration projects and other markets, logistically or economically at present, and that restoration specific culture facilities will be required to supplement the existing availability.

1.6.3. Management measures

In recent years management practices in the Solent have incorporated a minimum landing size (MLS) (70 mm ring) and limited fishing season (1st November - 28th February) (Southern IFCA, 2019). These measures were put in place to allow individuals to reach a size whereby, at the time of harvest, they would have reproduced at least once after reaching sexual maturity. First introduced by the authorities with Sea Fishery Committee responsibilities, the MLS was 50 mm and was later increased to 63 mm in 1976, at the same time restrictions on fishing season duration were introduced (Key & Davidson, 1981). Prior to this, it is likely that management loosely controlled the harvest and with no MLS this would have greatly reduced the chances of substantial and sustained reproduction on a scale to remain self-sustaining. The introduction of the Bottom Towed Fishing Gear 2016 byelaw by the Southern IFCA (Southern IFCA, 2019), covering a substantial area of the Solent (Fig. 1.8), is a significant step towards the protection of not only oyster populations, but all benthic habitats that are present. The byelaw is enforced year-round and presents an opportunity for restoration efforts.

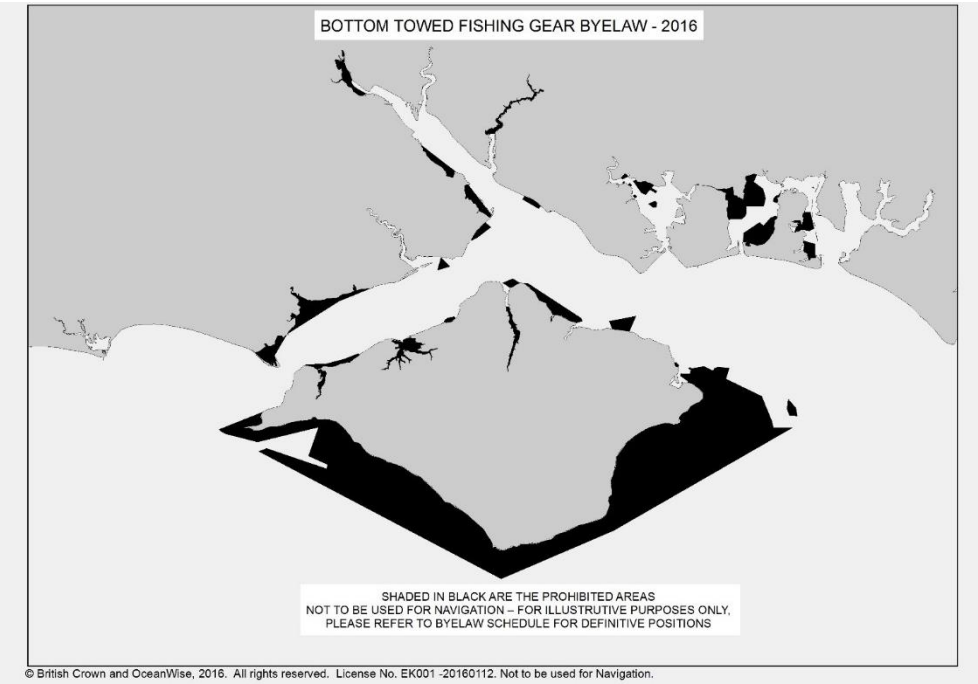


Figure 1.8. Bottom Towed Fishing Gear 2016 byelaw closure areas within the Solent introduced by the Southern Inshore Fisheries and Conservation Authority (Southern IFCA, 2019).

Alongside this additional protection is provided by the Solent Dredge Fishery Byelaw 2016 (Southern IFCA, 2019), prohibiting the use of a dredge within Southampton Water, Portsmouth Harbour and Langstone Harbour (Fig. 1.9) between 17:00 and 07:00 hours from 1st March to 31st October.

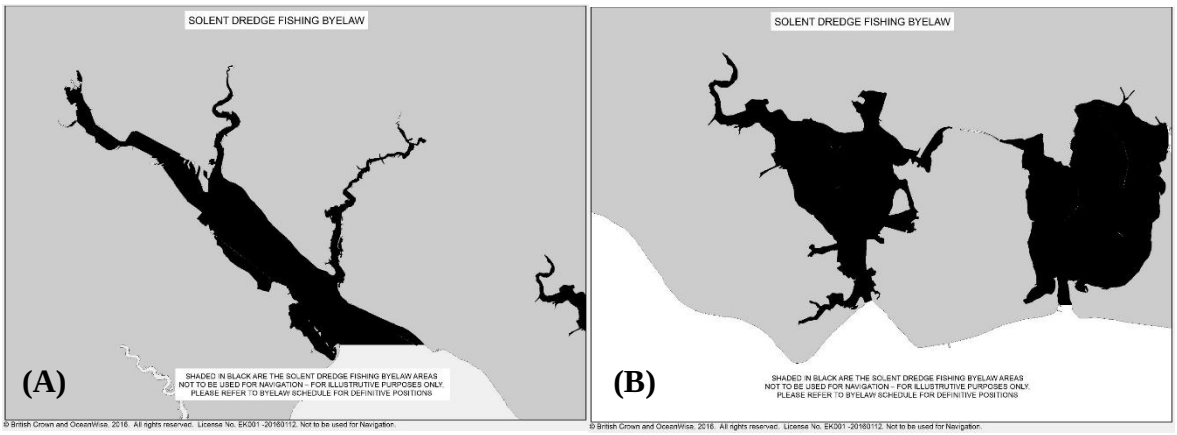


Figure 1.9. Solent Dredge Fishing Byelaw 2016 closure areas within (A) Southampton Water and (B) Portsmouth Harbour and Langstone Harbour introduced by the Southern Inshore Fisheries and Conservation Authority (Southern IFCA, 2019).

1.6.4. Invasive and introduced species

1.6.4.1. The American slipper limpet *Crepidula fornicata*

The presence and abundance of the invasive gastropod *Crepidula fornicata* is a major concern across Europe (Boyle, 1981; Blanchard, 1997), particularly in the Solent (Helmer *et al.*, 2019). The species was accidentally introduced with imports of *Crassostrea virginica* (Dodd, 1893; McMillan, 1938; Hoagland, 1985; Utting & Spencer, 1992; Minchin, McGrath & Duggan, 1995) and *Crassostrea gigas* (Blanchard, 1997) to Liverpool in the 1880s (Moore, 1880 in McMillan (1938)) and the east coast and Thames estuary in the 1890s and early 1900s (Crouch, 1893; Cole, 1915). Despite claims that C. *fornicata* may increase macrozoobenthic communities in muddy sediments (de Montaudouin & Sauriau, 1999), its rapid expansion throughout many areas of the UK (Orton, 1950; Chipperfield, 1951; Cole & Baird, 1953; Barnes, Goughlan & Holmes, 1973; Minchin, McGrath & Duggan, 1995) and Europe (Blanchard, 1997, 2009; Davis & Thompson, 2000; Thieltges, Strasser & Reise, 2003), including rapid colonization of oyster beds (Crouch, 1893), has serious ecological and economic impacts (see Blanchard, 1997). *Crepidula fornicata* has been shown to be detrimental to habitat suitability for juvenile fish (Le Pape, Guerault & Desaunay, 2004), suprabenthic biodiversity (Vallet et al., 2001), shell growth and survival of the commercially important bivalve *Mytilus edulis* (Thieltges, 2005). This invasive species is also attributed to habitat modification, through the production of mucoidal pseudofaeces, whereby benthic substrata change from predominantly sandy to muddy with a high organic content that rapidly becomes anoxic and unsuitable for other species (Streftaris & Zenetos, 2006). This negatively impacts the native oyster through a reduction in suitable substrata available for larval settlement (Blanchard, 1997), hindering recruitment and potentially oyster restoration efforts on the seabed.

1.6.4.2. The Pacific oyster *Crassostrea gigas*

There is divided opinion about the potential benefits and impacts of the introduced non-native species *Crassostrea gigas*. Predominantly distributed within the mid to low intertidal zone, *C. gigas* is tolerant of a range of habitats and is found in densities from 500 - 2000 / m^2 to 0.11 / m^2 (Kochmann *et al.*, 2013; Dolmer *et al.*, 2014). During its initial introduction to the UK, including areas such as Langstone Harbour (Askew, 1972) it was believed that temperatures were not high enough for *C. gigas* to reproduce (Spencer *et al.*, 1994; Child and Laing, 1998). This was not the case and populations are now well established (pers. obs.) and present in over 70 countries outside of its native waters, the northwest Pacific and Sea of Japan. It was introduced primarily for aquaculture purposes, with self-sustaining wild or feral populations present in 17 countries (Ruesink *et al.*, 2005).

Populations of *C. gigas* have recently been observed to overlap with populations of *O. edulis* in Scandinavia and Ireland (Laugen *et al.*, 2015; Zwerschke *et al.*, 2017), with the potential to displace the native species (Laugen *et al.*, 2015). Management of recently established *C. gigas* populations has been explored with some success (Guy and Roberts, 2010). They also have the capacity to co-occur in mixed subtidal reef structures with both *O. edulis* and *M. edulis* as seen along the Dutch coast (Zeeland) by Christianen *et al.* (2018). With limited populations of *O. edulis* remaining across much of Europe there is the opinion that *C. gigas* may provide alternative hard substrata required for larval settlement. However, the contrary opinion that the establishment of *C. gigas* populations is potentially harmful to the ecosystem as a whole is also expressed (Herbert *et al.*, 2016). Intertidal populations of *O. edulis* and *C. gigas* in sympatry have been observed recently in the Solent (J. Preston, pers. comm.; pers. obs.) indicating that ecological niche partitioning may exist, and cohabitation is possible. Further research is required to establish if the presence of *C. gigas* can indeed facilitate the recovery of *O. edulis* by providing a substratum, or a negative

species interaction is hindering recruitment. The influence on the benthic biodiversity associated with *C. gigas* is unclear, Guy *et al*. (2018) have shown *C. gigas* supports a lower epibiont species richness than *O. edulis* in the intertidal habitat, whereas Zwerschke *et al.* (2016, 2018) found similar species diversity and benthic assemblages with both species in the intertidal and subtidal environments, but, differences occurred between habitats and orientation. Positive effects on species richness and abundance in the presence of *C. gigas* reefs on bare rock and muddy substrata have also been observed (Lejart and Hily, 2011).

1.6.4.3. The American whelk tingle

Native to the Atlantic coast of North America (Franz, 1971) the American whelk tingle *Urosalpinx cinerea* is a predatory gastropod that feeds on a range of species and can occur in high densities (Walter, 1910; Galstoff *et al.* 1937), causing millions of dollars' worth of damage to fisheries (Hancock, 1954). The radula, in combination with the secretion of a softening agent from an accessory boring organ, is used to drill a circular hole through the valves of the prey before a proboscis is inserted to reach the flesh, which is then consumed using the radula (Boyle, 1981). This process can occur over a substantial period, two days for a 5 cm oyster and a week for a 10 cm oyster (Boyle, 1981). First recorded in the UK during 1927 amongst oyster populations in Essex (Orton and Winckworth, 1928) and later detected in samples preserved from 1920 (Orton, 1930), *U. cinerea* is currently limited in its spatial distribution to Kent and Essex. Mortalities in these areas were reported to exceed 50 % of the juvenile populations in 1953, with larger adults experiencing 10 % during the same year (Hancock, 1954). Assisted by cold winters in 1929 / 39 (Orton and Lewis, 1931; Cole 1942) *U. cinerea* completely outcompeted the native species *Ocenebra erinacea* (Hancock, 1954). At present *U. cinerea* is absent from the Solent and is not considered an immediate threat, however, the risk of accidental introduction remains a concern and should be considered before translocation of oysters takes place from the Kent and Essex populations.

1.6.5. Predation and pests

1.6.5.1. The European rough tingle *Ocenebra erinacea*

Natural levels of predation by native species has always occurred within *O. edulis* populations, however, the impact and predation levels has become more apparent as the populations have declined. The European rough tingle or European sting winkle *Ocenebra erinacea* is a species of predatory gastropod that is native to the UK and occurs primarily on the west and southwest coasts, including current and historically important oyster grounds in the Helford River, River Fal, West Mersea, and within the Solent (Orton, 1929; Laing *et al.*, 2005; Kamphausen, 2012). Orton (1929) highlighted the varying levels of predation, being high in areas where oysters were naturally abundant, and low in areas where the tingles have not established an appetite for oysters due to their absence.

The rough tingle can be present in great abundance, estimates made during 1976/77 indicate a substantial population of 30 million was present within the Solent (Key and Davidson, 1981). Lockwood (1985) estimated that 60 % of *O. edulis* mortalities within the Solent during the 1980s occurred as a result of *O. erinacea* drilling activity, seen after the population recovered from a crash due to the severe 1962/63 winter. This population of *O. erinacea* was severely depleted due to imposex caused by the introduction of Tributyltin (TBT) into antifouling paints (Hawkins and Hutchinson, 1990). In recent years, the opinion within the Solent fishing community is that there has been an increase in abundance of *O. erinacea*, and they are again causing significant mortalities within the oyster populations, research is ongoing to determine estimated abundances (Blue Marine Foundation, pers. comm.). Larvae have been shown to hatch in the veliger stage (Hawkins, 1985 in Lockwood, 1985) which may increase the speed of recovery after a population crash. The current predation risk, following the collapse of the *O. edulis* population, to oyster restored within the Solent is not yet fully understood and requires further investigation.

1.6.5.2. Other predators

A limited amount of predation is believed to occur within *O. edulis* populations from species such as the ommon seastar *Asterias rubens*, common sunstar *Crossaster papposus* (Hancock, 1955), *Carcinus maenas* (Mascaro & Seed, 2000) although Walne (1961) did not observe much predation from *C. maenas* on young spat. The larvae of *O. edulis* have also been detected within the stomach of conspecifics (Nelson, 1921; Korringa, 1941), but this is unlikely to be of consequence to the total population.

1.6.5.3. The boring sponge *Cliona celata*

Predation by *O. erinacea* can be mistaken for the presence of the boring sponge *Cliona celata*, a cosmopolitan species that bores into calcareous rock such as limestone and the shells of a variety of molluscs including *O. edulis*, *C. gigas* and *C. fornicata* (Fig. 1.10) (Hartman, 1957; Rosell *et al.*, 1999; Stefaniak *et al.*, 2005; Le Cam and Viard, 2011). Although the oscular papillae are conspicuous, the endolithic sponge could be likened to an iceberg with the tip exposed and the true extent of infestation occurring beneath the surface. *Cliona celata* can also occur in a larger form or raphyrus in a thick plate like structure but this is not considered to affect *O. edulis* and neither form feeds directly on host tissue.

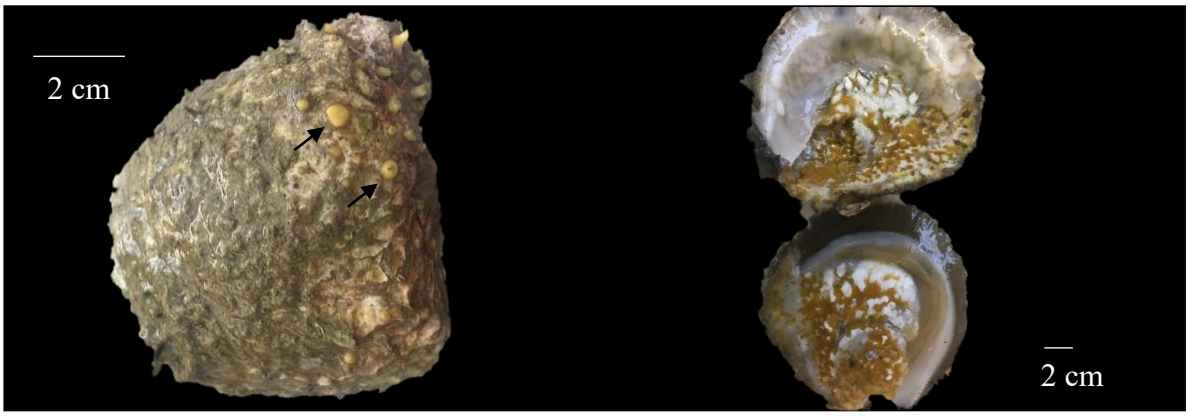


Figure 1.10. The external (left) and internal (right) extent of the boring sponge *Cliona celata* throughout the valves of a live *Ostrea edulis*. The papillae are observed on the extrnal surface of the shell (examples indicated by arrows) with the full extent of the organism revealed when the oyster is shucked to expose the internal structure.

The infiltration of *C. celata* leaves the valves of *O. edulis* brittle, fragile and vulnerable to predation, especially if the hinge or attachment point of the adductor muscle is affected (Hoeksema, 1983; Rosell *et al.*, 1999). Shells of deceased oysters are observed with small holes on the external that resemble the, often single, hole produced by *O. erinacea* in size and shape, others can become completely perforated by the sponge also allowing them to be distinguished from that of *O. erinacea*. Rosell *et al.* (1999) observed populations of *O. edulis* infested with *C. celata* in the Mediterranean Sea and found that the lower valve was always and exclusively affected, with the sponge extending throughout the shell to reach the inner side, an observation that was not made with *Littorina littorea* (Stefaniak *et al.*, 2005). *Cliona viridis* is also present in that region and also infest *O. edulis*.

If the valves are not damaged to the extent where the oyster is predated on, or the infestation is not excessive, then the metabolic activity required to secrete additional conchiolin to cover the holes may leave the individual with an energy deficiency in comparison to those not infested (Palmer, 1992), as seen in *L. littorea* (Stefaniak *et al.*, 2005). If the additional material is accreted on the internal surface then the volume of space for the internal anatomy of the mollusc is reduced, thus the body size and fecundity. However, this has been shown not to be the case for infested *C. fornicata* with minimal detrimental impacts (Le Cam and Viard, 2001).

Not only do these infestations leave *O. edulis* and other mollusc species vulnerable to predation but in commercial production areas they also have detrimental impacts on farmed stock or managed beds (Giard, 1881, Hoek 1887, 1902 in Korringa 1951b; Warburton, 1958; Wells, 1959; Thomas, 1979). There is limited information available that has determined the full extent of the impacts that *C. celata* inflicts upon *O. edulis* populations.

1.6.5.4. Other boring organisms

Two species of *Polydora* polychaete worms are known to infest the shells of *O. edulis*, the first being *Polydora hoplura* and the second *Polydora ciliata*. The former is regarded to have caused damage to the French oyster industry (Giard, 1881 in Korringa, 1951b) and penetrates the oyster shell internally in the area between the mantle and the, predominantly dorsal (flat), valve often opposite to the inhalant chamber (Korringa, 1951b). Once the worm has entered the valve it settles on the periphery and begins to accumulate sediment, this activity induces the oyster to secrete large green patches of conchiolin or a hard, calcareous layer. As the individual within the shell grows it punctures the material deposited and the oyster re-covers the area, a process that can occur multiple times until large mud filled blisters form covering the, often 'U' shaped, burrow (Korringa, 1951b).

Polydora ciliata, in contrast to *P. hoplura*, burrows between the scales of the, predominantly dorsal, valve (Korringa, 1951b) and in crevices around the hinge (pers. obs.). In extreme cases, mass mortalities have been observed with *P. ciliata* being the responsible organism (Le Loup, 1937, 1940 in Korringa, 1951b). Occasionally *P. ciliata* may burrow into the shell, with this occurring predominantly in the ventral valve and is understood to primarily occur when the worm requires shelter (Korringa, 1951b). A strong affinity of *P. ciliata* towards *O. edulis* over *C. gigas* has been observed in Plymouth Sound, although condition index (CI) differences within *O. edulis* could not be assessed due to 99 % infection, the worm presence did not affect the CI of *C. gigas* (Lemasson & Knights, 2019). Comparisons of infected and uninfected *O. edulis* would provide a valuable insight into the impact of *Polydora* infestation on shell integrity with implications for increased stress and predation risk and reduction in long-term survival.

1.6.6. Disease

The mortality and health problems associated with diseases are the single largest cause of economic losses in aquaculture globally (Meyer, 1991). Shellfish are susceptible to the detrimental effects of numerous parasitic organisms. Of particular concern for *O. edulis* is the impact of European Commission (EC) notifiable protozoan parasites within the genus *Bonamia* (Haplosporidia; Sprague 1979), especially *Bonamia ostreae*. The disease bonamiosis, caused by members of the genus of intrahaemocytic protozoan parasites *Bonamia*, including *B. ostreae*, has severely impacted *O. edulis* populations. The microcells (2 - 5 µm diameter) of *B. ostreae* enter into the haemocytes of the oysters by host-specified phagocytosis (Chagot *et al.*, 1992) and become systemic, overwhelm and eventually kill the infected individual. The increase in distribution of, and mass mortality events caused by, *B. ostreae*, since its introduction to Europe in the 1970s and 80s (MacKenzie *et al.*, 1997), are well documented (Figueras, 1991; Cigarria *et al.*, 1995, Laing *et al.*, 2005; Culloty and Mulcahy, 2007) with its impact as a non-native species driving disease emergence highlighted by Peeler *et al*. (2011).

Another member of the genus *B. exitiosa,* originating from the southern hemisphere in association with the host *O. chilensis* (Cranfield *et al.*, 2005) has subsequently been detected in *O. edulis* across continental Europe. The first detection occurred in 2006 (Galician coast, Spain [Abollo *et al*., 2008]), shortly followed by another in 2007 (Adriatic Sea, Italy [Narcisi *et al*., 2010]). The species has subsequently been detected in France (Mediterranean Sea [Arzul *et al*., 2010]), the Spanish Mediterranean coast (Carrasco *et al.*, 2012), Britain (Cornwall [Longshaw *et al*., 2013]) and Portugal (Algarve [Batista *et al*., 2016]). The first UK population, in which *B. exitiosa* was detected, was in the River Fal, Cornwall (Longshaw *et al*., 2013), 28 years after the first diagnosis of *B. ostreae* in the UK, also in the River Fal (Bucke and Feist, 1985; Hudson and Hill 1991). To date there have been no reported mass

mortality events where *B. exitiosa* has been considered the aetiological agent and detection has been within a limited number of individuals within the sampled populations.

Another concern is Aber disease or Marteiliosis, which is caused by another protozoan parasite, within the order Paramyxida, *Marteilia refringens* (Berthe *et al.*, 2004). As with *B. ostreae*, *M. refringens* is listed as a European Commission (EC) notifiable protozoan parasite and has been observed in *O. edulis*, *Mytilus edulis* and *M. galloprovincialis* populations across Europe, described as the etiological agent responsible for mass mortality events (Herrbach, 1971; Grizel, *et al*. 1974; Alderman, 1979; Grizel, 1985 in Berthe *et al.*, 2004; Villalba *et al.*, 1993; Figueras and Montes, 1988; López-Sanmartín *et al.*, 2015). The River Tamar is currently the only UK waterbody that has been designated for the presence of *M. refringens* (CEFAS, 2011). Recent assessments did not detect any evidence of O-type (*M. refringens*) infection in *O. edulis* from the UK (Tamar estuary), Sweden and Norway. M-type (*Marteilia pararefringens*) has been detected in *M. edulis* populations that were in close proximity but was absent from *O. edulis* (Kerr *et al.*, 2018).

1.6.7. Water quality

A plethora of factors contribute to the poor water quality of the Solent, especially within Southampton Water, Portsmouth, Langstone and Chichester Harbours (Environment Agency, 2016a, b; Williams and Davies, 2018). Human population growth and the associated coastal development has increased the quantity of wastewater produced and contaminants contained within run-off. Much of the sewage systems in the cities are outdated and combine with storm water to an extent that overrun the wastewater treatment plants current systems (Langstone Harbour Board, 2019). The level of disregard for the environmental conditions from the water company responsible for the catchment area, Southern Water Services Limited, is highlighted by the £126 million penalty proposal

imposed by the regulatory body Ofwat. This penalty is the result of deliberate misreporting of information and insufficient investments which led to failures and spills of wastewater (Ofwat, 2019). The severity is emphasised by the comments made by the chief executive of Ofwat:

"What we found in this case is shocking. In all, it shows the company was being run with scant regard for its responsibilities to society and the environment. It was not just the poor operational performance but the coordinated efforts to hide and deceive customers of the fact that are so troubling."

The excessive quantities of wastewater are further exacerbated by the input of nutrients from agricultural runoff. The resulting enrichment of water by nitrogen compounds causes accelerated algal growth and higher forms of plant life, in turn disturbing the balance of organisms present in the water. Once these organisms degrade, the area becomes anoxic, forming dead zones. The levels of eutrophication are monitored by the Environment Agency (EA) who have designated the areas surrounding the Hamble Estuary, Portsmouth Harbour, Langstone Harbour and Chichester Harbour as nitrate vulnerable zones (NVZs) and the water bodies as sensitive (eutrophic) and polluted (eutrophic) areas (Environment Agency, 2016a, b). The visual evidence of the ecological impact this has on the areas can be seen by the widespread presence of opportunistic macroalgae that smothers the intertidal mudflat and saltmarsh habitats (pers. obs.). The economic and health impacts are also evident as the fishery, for human consumption, is classified depending on the levels of *E. coli* within the oysters. To comply with the Water Framework Directive (WFD) 75 % of samples need to record ≤ 300 *E. coli* / 100 g shellfish flesh. The Thorney Channel in Chichester Harbour was closed for the fishing season in 2018 due to shellfish quality results failing to comply with these requirements (Sussex IFCA, 2018a).

Water quality in the Solent is also subject to contamination from other anthropogenic sources such as Tributyltin (TBT), one of the most toxic substances previously used in antifouling paints and particularly potent for marine molluscs including *O. edulis* (Thain and Waldock, 1986; Thain *et al.* 1986; Axiak *et al.*, 1995; Laing *et al.* 2005). The Solent is one of the busiest waterways in the UK with intense recreational, commercial / industrial and naval shipping activity occurring along the majority of the coastline and the quantity of TBT applied during the period of legal use was considerable. Despite initial bans introduced in 1987, followed by a complete ban in 2008 (Gipperth, 2009) numerous studies have detected TBT in high concentrations within sediments in recent years and indications suggest it can remain within the environment for up to 30 years (Champ *et al.*, 1996; Radke *et al.*, 2018; Filipkowska and Kowalewska, 2019). This is of particular concern with the intense levels of maintenance dredging that occurs in the Solent and the capital dredge of the entrance to Portsmouth Harbour that took place from 2016 - 2017 (Hopper, 2017). Resuspension of TBT could cause failures in the reproduction of *O. edulis* as transition of adults from male to female state, larval production, larvae and development of recently settled spat have been shown to be affected by TBT concentrations in comparison to unexposed populations (Thain and Waldock, 1986).

Other pollutants such as heavy metals, organic pesticides, polychlorinated biphenyl (PCB) and polycyclic aromatic hydrocarbons (PAHs) are known to affect the adults and larval of multiple mollusc species (Calabrese *et al.*, 1977; Gagnaire *et al.*, 2006, 2007; Girón-Pérez, 2010). The effects of heavy metals, especially Copper and Cadmium, are associated with increased mortality and decreased phagocytic activity in *O. edulis* (Auffret *et al.*, 2002) and inhibition of filtration rates, altered sex ratios towards male dominated populations and reduction in gonado-somatic in blood clams *Tegillarca granosa* (Liu *et al.*, 2014). This immunosuppression described by Auffret *et al.* (2002) may be linked to increases in

pathogen, such as *B. ostreae*, susceptibility and in an area such as the Solent, where heavy metal contamination occurs (Leatherland and Burton, 1974; Oyenekan, 1981 in Lockwood, 1985), this should be taken into consideration when selecting suitable restoration locations.

1.6.8. Climatic conditions and severe weather events

Marine and terrestrial environments are subject to periods of extreme climatic conditions that can have devastating effects on certain populations that are less resilient to these stressors. With current projections of global climate change and ocean acidification (IPCC, 2018) the frequency of these events and therefore the stressors on marine life are likely to increase. Indications suggest that *O. edulis* populations will be better adapted to these predicted climatic conditions than *C. gigas* with regards to metabolic rate, feeding rate and somatic growth (Lemasson *et al.*, 2018) and sustained higher temperatures, of 21 °C, may prove beneficial for *O. edulis* (Mann, 1979). Despite this, environmental changes with regards to ocean acidification are expected to be detrimental to both species as they rely on calcification of shell material (Lemasson *et al.*, 2017). Increases in sea surface temperature (SST) may however facilitate range expansion of *C. gigas*. Global climate change not only induces warmer periods, but colder periods also. Marine organisms around the UK, including populations of *O. edulis*, were subject to such events during the winters of 1939/40 and 1962/3 where sea temperatures dropped to 0°C and resulting mortality reached 70 - 100 % in Essex, 50 - 100 % along the South coast and 30 % in Jersey (Cole, 1940; Orton, 1940; Crisp, 1964; Waugh, 1964). Similar events occurred in 2018, causing mass mortality of marine life. This should also be a consideration for restoration attempts that do not wish to incur significant mortalities in coastal areas.

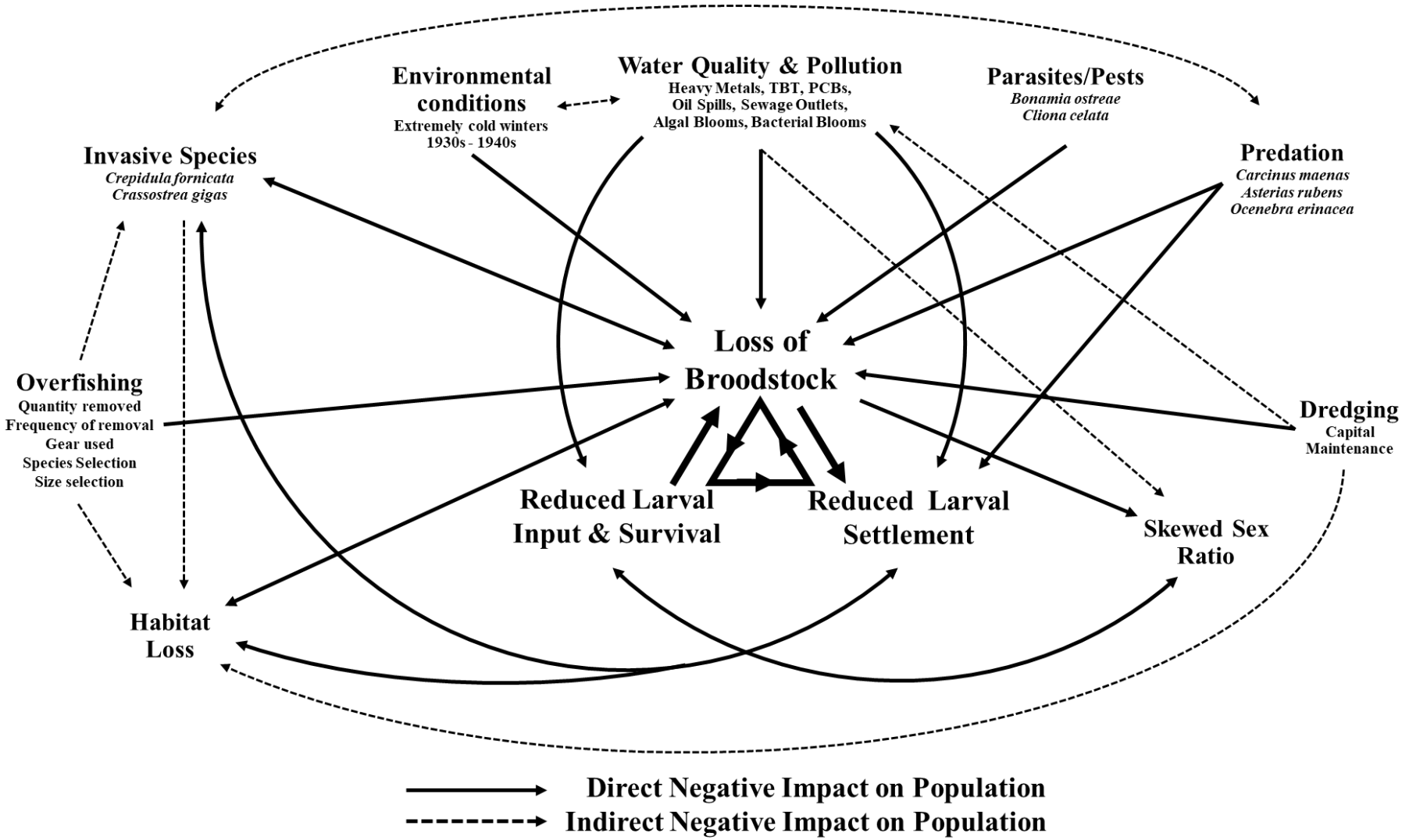
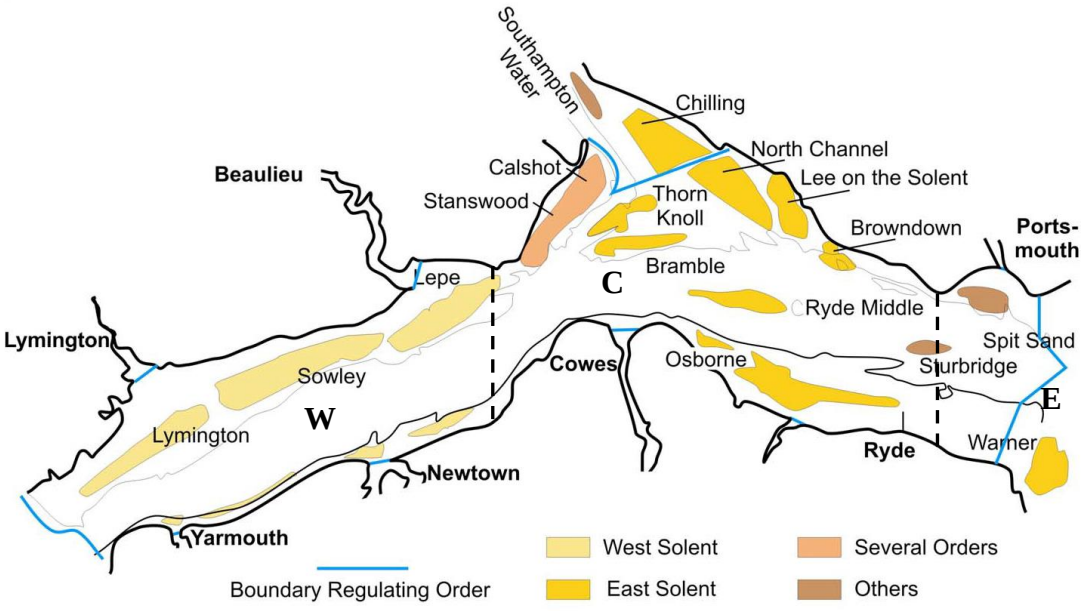


Figure 1.11. The factors that are currently known to be adversely affecting *Ostrea edulis* populations within the Solent and their interconnecting relationships. Examples of the factors are shown where necessary.

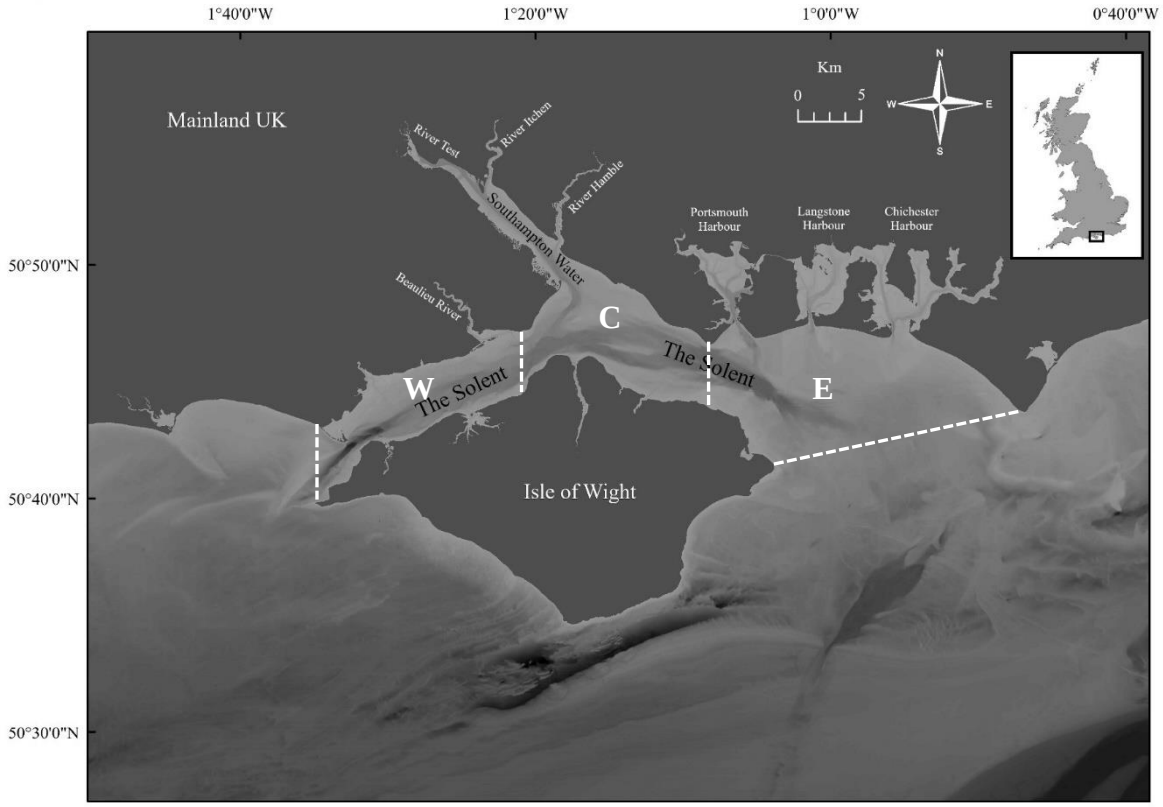
1.7. The Solent environment for restoration purposes

Restoration projects, including that operating within the Solent, are aiming to focus efforts on areas that not only historically supported substantial oyster populations, but also currently have suitable environmental conditions to support the relaying and survival of oysters at a variety of ages. For the purposes of this section, recent populations of *O. edulis* that have been depleted, shown in Figure 1.12A, will be described in the western, central and eastern areas of the Solent, as denoted by Figure 1.12B. There is little or no evidence in recent history of any considerable *O. edulis* beds or reefs on the southern coastline of the Isle of Wight other than those in the centre of the English Channel depicted by Olsen (1883), it is not clear if this is due to lack of presence or lack of surveying in that area. The majority of the areas described in this section are now barren and contain very few oysters (Vanstaen and Palmer 2010; Kamphausen, 2012; CEFAS, 2013; Southern IFCA, 2015, 2017, 2018).

It is likely that restoration efforts will need to focus activities on a limited number of these areas that historically supported *O. edulis* populations with the intent of protecting and preparing the benthic environment in others that are likely to be populated by larval dispersal. Thorough investigations into the current state of the substrata present and environmental conditions will allow appropriate management decisions to be made on how best to proceed with future relaying or cultch deployment activities.



(A)



(B)

Figure 1.12. (A) Historical locations of oyster beds within the Solent and (B) the wider Solent, separated into the western (**W**), central (**C**) and eastern (**E**) areas, separated by dashed lines with each section incorporating all water bodies. Map created using ArcMap software. Areas shown are irrespective of any authority or legislative boundaries. Source (A): Palmer and Firmin (2011).

1.7.1. The western Solent

Historically it was believed that considerable populations of *O. edulis* situated within the western areas of the Solent, such as Lymington, Beaulieu and Newtown, were responsible for the successful settlement seen in the central and eastern areas of the Solent. These populations experienced large mortalities due to an unknown cause during the severe winter of 1962/63, however, those remaining in the Beaulieu River produced significant quantities of larvae that are believed to be responsible for the successful spall fall observed in 1969 (Key and Davidson, 1981). The surviving recruits from that cohort were centred in the area around Lepe Middle Ground as well as Stanswood and Calshot, reaching a size of 35 mm by 1976 (Key and Davidson, 1981; Lockwood, 1985). The main fishery areas during 1975, 1977 and 1979 also incorporated the majority of the coastline north of the main channel from Lymington to Beaulieu and south of the main channel from Yarmouth to Newtown and from Newtown to West Cowes. These areas predominantly recorded catch rates of 1 - 15 oysters per haul, with a proportion of the beds reaching > 15 oysters per haul (Key and Davidson, 1981). These areas were extensively surveyed in 1980, much of Lymington Bank contained 1 - 49 oysters per haul, with one haul containing > 49 oysters. Sowley Ground also contained vast areas of oysters the provided hauls in excess of 15 oysters per haul. Lepe Middle Ground predominantly provided hauls of 1 - 5 oysters per haul. On the southern side of the main channel all areas provided < 49 oysters per haul, but many contained > 6 per haul. These areas were determined to be dominated by gravel-based substratum. The large quantities of juvenile settlement, seen in 1975, occurred mainly on stones and gravel. If the benthic environment remains as it was reported then the western Solent could provide valuable areas for restoration activities to take place, with the understanding that tingle predation may be an issue in this area (Key and Davidson, 1981).

1.7.2. Central Solent

Numerous oyster beds existed within the central Solent region and by far one of the most successful areas for recurrent settlement was the, previously mentioned, area stretching from Stanswood to Calshot. Records show that it was the main area fished for oysters in 1972 and catch rates of > 15 oysters per haul were obtained across the whole region. Large proportions of the area also reported catch rates of > 15 oysters per haul in 1975, 1977 and 1979, with catch rates of 1 - 15 oysters per haul obtained for the remainder of the area for all years (Key and Davidson, 1981). These areas were extensively surveyed in 1980 with the majority of the dredge hauls in Stanswood Bay and Calshot containing > 49 oysters. Dominated by sand and gravel with substantial quantities of oyster and *C. fornicata* shell, it is clear that the benthic environment provided ideal conditions for the large settlement of *O. edulis*.

Significant areas of oyster beds were recorded in Bramble Bank, Thorn Knoll, Southampton Water, Chilling, North Channel, Lee-on-the-Solent (Browndown Bank), Ryde Middle, Osbourne Bay and Mother Bank (Key and Davidson, 1981). From 1975 - 1979 these beds consisted of large areas were hauls contained 1 - 15 oysters with smaller pockets that contained > 15 oysters. During the 1980 survey Bramble Bank, Ryde Middle, and Browndown Bank were the most productive containing in excess of 15 oysters per haul, the other areas consistently contained 1 - 14 oysters per haul. Much of these beds occurred on a mud-dominated substratum with vast areas of *C. fornicata* shell interconnecting the beds of live oysters and deceased oyster shells.

It is clear that Stanswood Bay should be the focus of restoration activities in the central Solent, many of the other beds were less productive and are situated within an extremely busy shipping channel which may pose logistical issues. However, they could provide areas for relaying of cultch to achieve spatfall from a potential Stanswood population.

1.7.3. The eastern Solent

One of the first mentions of oyster related activities in the eastern Solent is from the South of England Oyster Company (1865) who proposed to introduce oysters into Langstone Harbour in an effort to increase spatfall by creating an artificial breeding ground. Records indicate that 3 million spat were produced in one pond in 1867 (Sullivan, 1870) and that during the 1972/73 season 15,000 oysters per month were landed from the wild fishery within the Harbours (Davidson, 1976). Key and Davidson (1981) unfortunately provide limited information on the eastern Harbours at this point in time, however, a reasonably sized bed is described in the Warner Bank area and appears in the fishery record during 1977, 1999 and 1980. The area recorded hauls of 1 - 15 oysters with smaller patches of > 15 oysters per haul, with the in-depth 1980 survey showing catches of 1 - 14 oysters in all but one haul, where between 15 - 49 oysters were caught. No information was provided on the substratum for the area. The substratum around Spitsand, at the entrance to Portsmouth Harbour, is described as mud and sand for the majority of the area, with a large area of *Crepidula* shell also present, however, no values are provided for the quantities of oysters in this area. The area of Sturbridge is mentioned as an area for oyster production in 1979, where almost the whole area provided hauls of 1 - 15 oysters.

In recent years, the main productive areas within the Harbours have been Fareham Creek and Porchester Channel in Portsmouth Harbour, Langstone Main Channel in Langstone Harbour, and Emsworth Channel and Throney Channel in Chichester Harbour (Southern IFCA, 2015, 2017, 2018; Sussex IFCA, 2017).

1.8. Thesis aims and objectives

With the current impoverished benthic environment and degraded environmental conditions, anthropogenic activities are required to allow for successful restoration of *Ostrea edulis* populations in the Solent and across Europe. The Solent area appears to be both substrate and recruitment limited, as well as having unfavourable water quality, therefore, future efforts should be made to address these issues with a focus on oyster survival, growth, reproduction and recruitment. By restoring oysters in sufficient quantities many of the issues currently faced are in turn likely to be mitigated against and allow for self-sustaining populations to establish, forming a circular oyster economy. With the correct site selection, protection, legislation and enforcement these populations, intended for ecological restoration purposes, could in-turn spill over into sustainably managed fishery areas.

There were multiple aims of this research project that was conducted as part of the Solent Oyster Restoration Project, founded by the Blue Marine Foundation (www.bluemarinefoundation.com/project/solent/). Understanding the current population demographics and distribution within the eastern Solent was a key objective of this study as for future restoration metrics to be meaningful, a baseline is required, even if this is considered a continuation of a sliding baseline from historic populations. Relating this to known invasive species also provides detail on ecological shifts that may be occurring.

Furthermore, the study focused on the development of a novel solution to address reported recruitment failure and dwindling broodstock populations. At the time of the study the fishery was not regulated in a sustainable manner and the implementation of the previously mentioned bylaws was a work in progress. Large enough quantities of oysters were not available for any meaningful relaying attempts and the legislative process involved for restoration project to obtain licences to conduct such work is currently excessive, expensive, time consuming and laborious, therefore no permissions were in place to attempt

either live oyster or cultch laying. By utilising existing floating structures these issues were avoided and provided an opportunity to develop a system that could be implemented whilst the issues previously mentioned were being addressed. By suspending broodstock populations beneath marina pontoons, with the intention of them acting as “larval pumps”, no licences were required (registration as an “aquaculture facility” was however). Oysters were protected from fishery activity and to an extent, predation, and issues surrounding access were reduced as the systems were not as limited by weather conditions or the requirement for diver or benthic surveying.

The specific aims of the first section of this study were to:

1. Evaluate the current densities and distribution of *Ostrea edulis* in relation to *Crepidula fornicata* in the Solent region;
    a. Determine the changes in density and distribution of *O. edulis* and *C. fornicata* over a substantial time period and assess any shifts in populations in the Solent;
    b. Assess multiple years of Solent fishery data to determine any trends in population demographics, and;
    c. Assess any alterations to the reported skewed sex ratio within benthic populations.
2. Determine the efficacy of suspended broodstock cages with regards to adult mortality and condition in two densities under different cleaning regimes across a localised spatial range.
    a. Define environmental limits for broodstock cage systems; and provide recommendations for suspended cages as broodstock systems in restoration projects.
3. Determine timing and extent of reproduction in these adult populations in elevated conditions across a localised spatial range;
    a. Determine if larval recruitment was successful in areas where broodstock cages were introduced and in relatively close proximity to broodstock populations, and;
    b. Determine relative larval abundances within the water column in relation to *Crassostrea gigas* and *C. fornicata*.
4. Assess the changes in pathogen, *Bonamia ostreae* and *B. exitiosa*, prevalence within densely congregated broodstock oyster populations and any larval broods.

Figures 1.13 and 1.14 outline the aims of each section of this study.

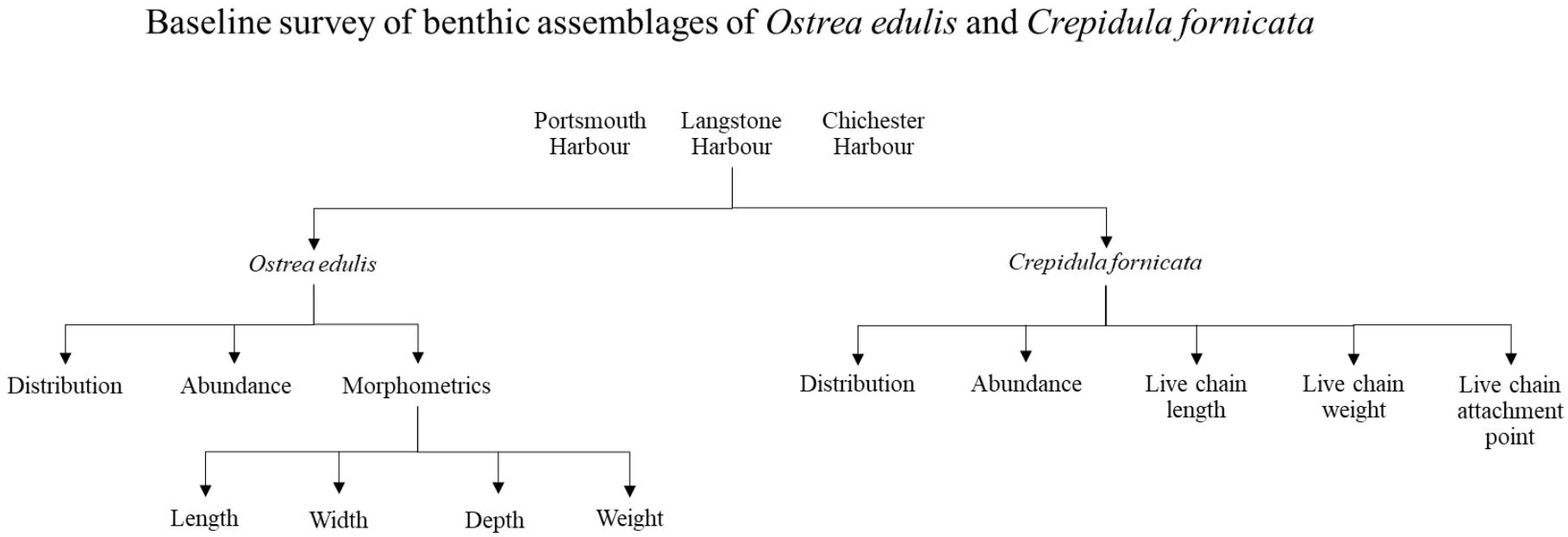


Figure 1.13. Schematic outlining the various parameters that were assessed.

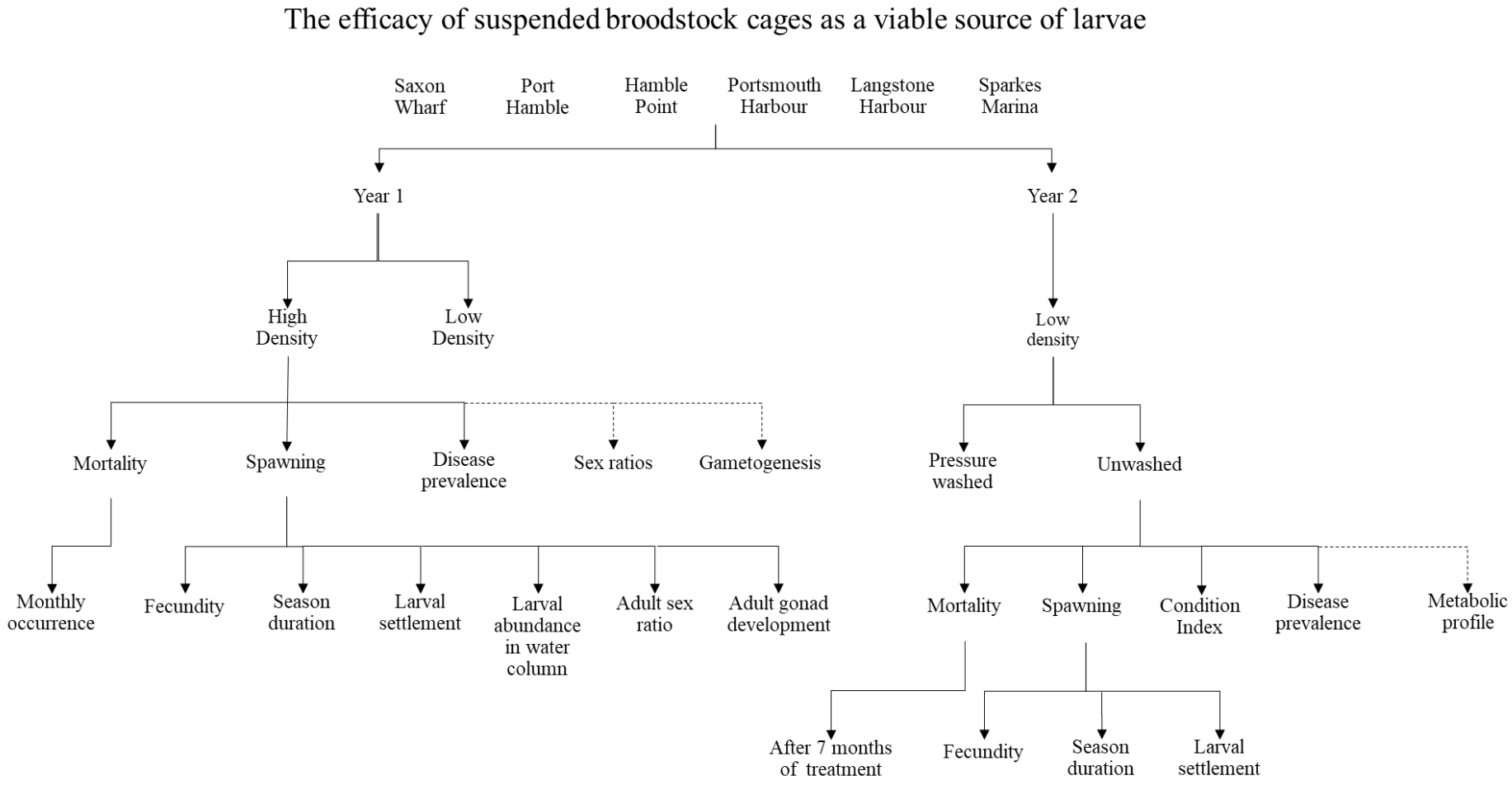


Figure 1.14. Schematic outlining the various parameters that were assessed in the second section of this thesis. Dashed lines indicate sections where samples were collected and preserved but that could not be analysed.

Chapter 2

Assessment of the Current Benthic Community of Native Oysters and Invasive Slipper Limpets

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2.1. Introduction

With an 85 % loss of the global oyster populations, many reefs and beds are now considered to be functionally extinct, whereby the individuals no longer play a significant role in the ecosystem functioning, or the reproductive capability of the population is no longer viable (Beck *et al*., 2011). To begin the investigation into the efficacy of restoration efforts within the Solent, this chapter presents an assessment of the essential baseline data regarding the current benthic assemblages of both the European flat oyster *Ostrea edulis* and the invasive American slipper limpet *Crepidula fornicata*, known to be abundant in the area, in three eastern Solent Harbours. The information provided by this study will allow for the quantification of restoration metrics used to assess the success of projects in their local area and across Europe in a similar manner to that which has been conducted for *Crassostrea virginica* in the USA (zu Ermgassen *et al.*, 2016). This chapter tests the hypothesis that oyster populations within the eastern Solent harbours are at the point of functional extinction and that an ecological phase shift has occurred, where *C. fornicata* has occupied the ecological niche that has arisen as a result of the removal of *O. edulis*. Temporal data for the populations, from nearly 20 years ago, was available for Chichester Harbour and this was used to determine how drastic the shift has been in the recent history of the area. The impact the fishery has had on population demographics during the past few years was also assessed in order to determine how recruitment and age classes were impacted. The condition and disease status of oysters within the three harbours was also determined, with the disease status related to previously recorded levels. The data collated in this study and others allows an assessment into the availability of suitable substrata for future larval recruitment and will determine areas that require additional cultch material, or limpet removal, to facilitating this settlement.

2.2. Methods

2.2.1. Benthic surveys of *Ostrea edulis* and *Crepidula fornicata* densities and distribution in the eastern Solent Harbours: 2017

The harbours of Portsmouth, Langstone and Chichester were selected for the analysis of this study due to their historically abundant fisheries and accessibility. Within each harbour 30 to 31 precise subtidal locations were chosen (Fig. 2.1) and at each of these locations three replicate samples were collected using a 0.1 m^2 Van Veen grab on board the 10.67 m research vessel (RV) Chinook II (Offshore 105 Pilothouse). This method was chosen, over the use of a dredge, as it was known that large quanitites of *C. fornicata* were present in many of the areas and the grab allowed for these to be accurately quantitative. If the small dredge, that was available to the researcher, were to be used it would have filled quickly and at an unknown point, with excess *C. fornicata* spilling out and not accounted for (Key and Davidson, 1981). In addition, the recent and regular surveys conducted by the Souther Inshore Fisheries and Conservation Authority (IFCA), that employ a dredge, provide a relatively comprehensive indication overview of existing location of the few remaining populations but do not account for *C. fornicata*.

Benthic surveys occurred before the opening of the 2017 fishing season for areas that were to be actively fished for oysters. They were conducted during February in Langstone Harbour, April and October in Portsmouth Harbour and October and December in Chichester Harbour. The 2017 active fishery area of Chichester Harbour (Emsworth Channel, E, Fig. 2.2) was sampled before opening on the 1st November 2017. The remaining areas of the harbour were sampled subsequently as they were not opened as active fishery areas, due to oysters either being unfit for human consumption with high recordings of *E. coli* or being voluntary broodstock protection areas under agreements with the local fishermen (Sussex IFCA, 2018). For each sample all material collected was passed through a 6 mm square mesh

box sieve, to remove excess sediment, and placed into individually sealed and labelled plastic bags when onboard the RV. Samples were then returned to the laboratory where they were rinsed and passed through a 6 mm square mesh box sieve for a second time to remove any remaining sediment, allowing for observations of live organisms with ease. Total oyster (*O. edulis*) and limpet (*C. fornicata*) densities were recorded for each sample location along with the chain length of live limpets, chain biomass of live limpets and the attachment substratum for each individual chain. Chain length, thus chain biomass, was determined as the total number of individual *C. fornicata* attached to one another in a single mass, irrespective of the direction of attachment and excluding any deceased shells used as attachment substratum. Chains were considered to be separate when the substratum had multiple chains attached to it and these chains were not interconnected by live individuals. Any attachment substratum that was not a live limpet was removed prior to biomass recordings. Geographical position of each sampling location was assessed with a precision of 2 m using a Lowrance® Elite 7m GPS system aboard the RV. See Appendix A for exact sample location coordinates. Distribution and abundance of oysters and slipper limpets were successfully surveyed at all sites within all three harbours.

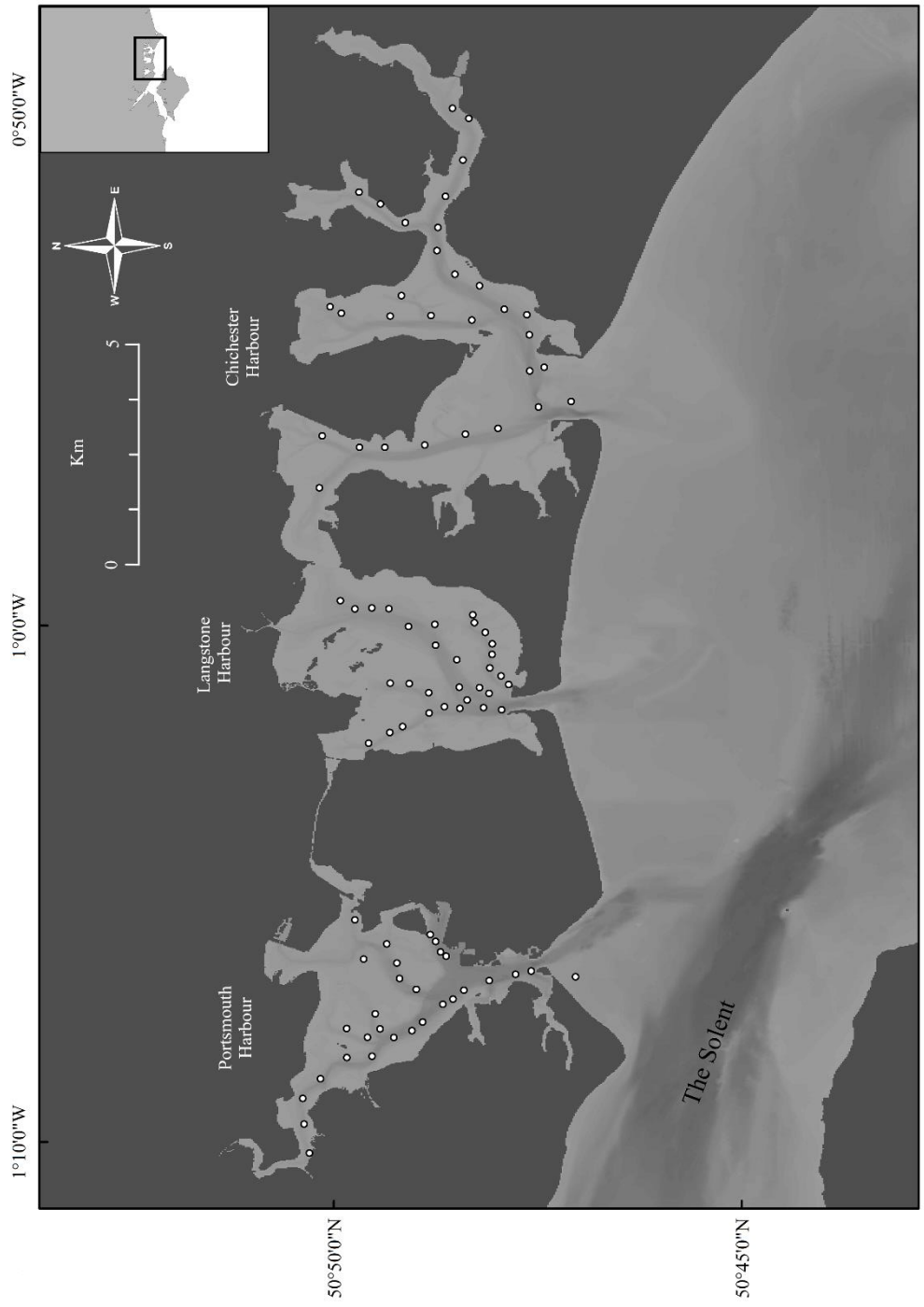


Figure 2.1. The wider Solent, showing the benthic sample locations within Portsmouth, Langstone and Chichester harbours for the 2017 survey, three 0.1 m^2 samples were retrieved from each area marked by a ○ with areas selected to cover the maximum amount of each harbour within reason. Map created using ArcMap software.

2.2.2. Comparison of *Ostrea edulis* and *Crepidula fornicata* benthic surveys between 1998 and 2017 within Chichester Harbour

The distribution and abundance of *O. edulis* and *C. fornicata*, among other species, was recorded 19 years prior to the current study by Farrell (1998). A box dredge, on board research vessel (RV) Thrift, was used to sample 26 of 31 intended locations, three of these locations were inaccessible at the time and were later hand dug at extreme low water spring tide and the final two intended locations could not be sampled due to other logistical reasons. Sample area was calculated as follows:

Dredge volume = 36 litres, = 36000 cm^3

Mean thickness of sediment layer = 6 cm

Theoretical area sampled by full dredge = 36000/6, = 6000 cm^2, = 0.6 m^2

These locations were again selected for the current study in order to get a direct temporal comparison of the distribution and abundance of *O. edulis* and *C. fornicata* at 29 of the 31 proposed sites within Chichester Harbour. All 31 of the intended sites were successfully sampled during the 2017 survey using the 0.1 m^2 Van Veen grab, as previously mentioned, on board RV Chinook II. Despite differences in the sampling methodology both studies provided results in comparable measurements of individuals / m^2 allowing for a direct comparison to be obtained.

2.2.3. Oyster population demographics within Portsmouth, Langstone and Chichester Harbours

Demographic population data from 2015 - 2017 were derived from oysters captured by commissioned dredge fishing at the beginning of the open fishing season, with no selection for minimum landing size. All oysters were collected using ladder dredges in accordance with the local byelaw conditions put in place by the relevant Inshore Fisheries and Conservation Authorities (IFCAs) for Portsmouth and Langstone harbours (Southern IFCA, 2018a) and Chichester Harbour (Sussex IFCA, 2018). Oysters were obtained from commercial fishery areas near the entrance of Portsmouth Harbour (Hamilton Bank and Spitsand, H+S, Fig. 2.2) and within Chichester Harbour (Emsworth and Thorney Channels, E & T, Fig. 2.2) during November 2015. Oysters from Langstone Harbour fishery (Sinah Lake and Langstone Channel, S & L, Fig. 2.2) were also obtained during November 2016. Further samples of oysters from Langstone (Sinah Lake and Langstone Channel, S & L, Fig. 2.2) and Chichester (Emsworth Channel, E, Fig. 2.2) harbours were obtained during 2017, but unfortunately, the fishermen mixed the two harbour populations sampled on landing. Live oysters were cleaned to remove epibionts and blotted dry before measuring. Measurements (Fig. 2.3) for the maximum shell length, maximum shell width, maximum shell depth (mm) and whole wet weight (g) were recorded for a minimum of 700 oysters from each harbour. Maximum shell depth was not recorded for the first 500 Chichester and Portsmouth oysters, but was recorded for the final 200 individuals. The attachment point, predominantly around the umbo / hinge area, was recorded for every oyster as either 1) *Crepidula fornicata* shell (always dead), 2) oyster shell (alive or dead), or 3) absent, whereby no evidence of the attachment point or material was clearly observed or left in place after the dredging process.

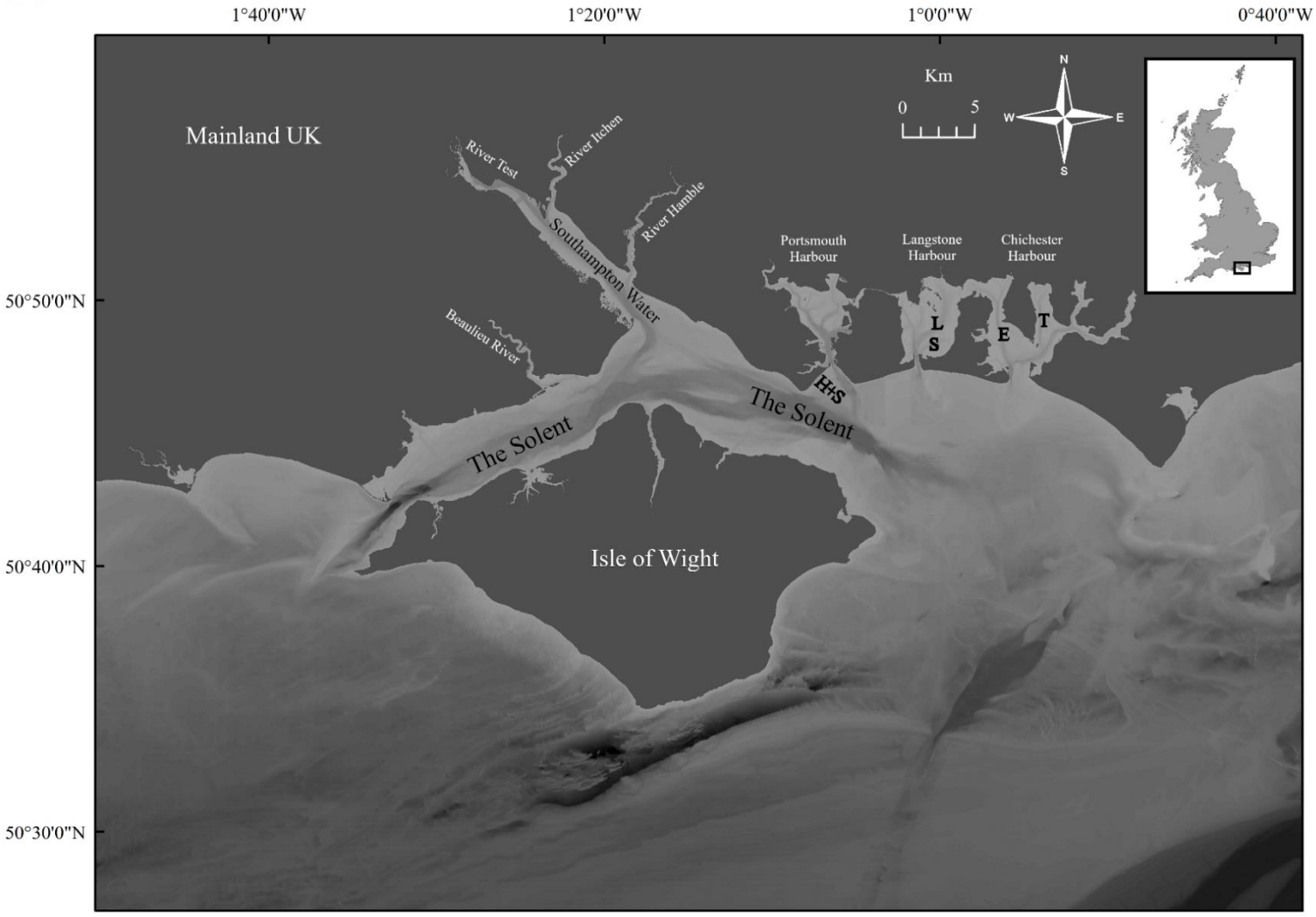


Figure 2.2. The wider Solent, showing the three harbours under investigation with locations of sample collection. (H+S) Hamilton Bank and Spitsand, (S) Sinah Lake, (L) Langstone Channel, (E) Emsworth Channel, (T) Thorney Channel. Map created using ArcMap software.

Figure 2.3. Morphometric measurements recorded for *Ostrea edulis*. Maximum shell length, width and depth (mm) shown by the respective directional arrows.

2.2.4. Condition index and prevalence of the parasite *Bonamia ostreae* within fishery oysters

Oysters sampled for screening were immediately frozen and stored at -20 °C. Analysis of condition index (CI) was performed to compare 2015 Chichester (n = 24) and 2015 Portsmouth (n = 24) oyster populations according to the methodology in Culloty *et al.* (2004, p 45) with modifications, 105 °C for 24 h opposed to 60 °C for 48 h. The calculation used by Walne & Mann (1975) and Lucas & Beninger (1985) (cited in Culloty *et al.*, (2004) was used to determine CI:

Condition index = *Dry Tissue Weight* / *Dry Shell Weight* x 100.

Gill tissue samples were taken prior to CI analysis, in addition a further 24 oysters were selected at random from the landings of both harbours to determine the presence of *Bonamia ostreae* within the population. For each specimen a 5 mm section of gill tissue was removed with a sterile blade and genomic DNA extractions were performed using DNeasy® Blood & Tissue kits (QIAGEN™, Germany) following the tissue protocol provided by the manufacturer. Quantification of the DNA extractions was conducted using a NanoDrop® 1000 Spectrophotometer (NanoDrop®, Thermo Fisher Scientific Inc., USA). Species-

specific primers Oe fw_1 + Oe rev_4 (Gercken & Schmidt, 2014) were used to amplify the cytochrome c oxidase subunit I (*coxI*) gene from the oyster DNA, as a positive control. Species-specific primer pairs BO + BOAS (Cochennec *et al.*, 2000), BOSTRE-F + BOSTRE-R (Ramilo *et al.*, 2013) and C_F + C_R (Carnegie *et al.*, 2000) were used to amplify *B. ostreae* SSU rRNA, 18S-ITS 1 rRNA and 18S-ITS 1 rRNA, respectively (Table 2.1).

Table 2.1. Primer sequences used in this chapter to confirm identification of *Ostrea edulis* and detect *Bonamia ostreae* DNA present within the gill tissue of the oysters.

Species	Primer pair	Primer sequences	Amplicon size (bp)	Reference
Ostrea edulis coxI	Oe fw_1	5'-ATG-GGA-CGA-TTT-GAT-AGA-GC-3'	1100	(Gercken & Schmidt, 2014)
	Oe rev_4	5'-CCC-AAA-TAA-CGG-GAA-AAG-TGC-TAA-CCA-CCA-GAA-TGA-3'		
Bonamia ostreae SSU	BO	5'-CAT-TTA-ATT-GGT-CGG-GCC-GC-3'	300	(Cochennec *et al.*, 2000)
	BOAS	5'-CTG-ATC-GTC-TTC-GAT-CCC-CC-3'		
Bonamia ostreae 18S-ITS 1	BOSTRE-F	5'-TTA-CGT-CCC-TGC-CCT-TTG-TA-3'	208	(Ramilo *et al.*, 2013)
	BOSTRE-R	5'-TCG-CGG-TTG-AAT-TTT-ATC-GT -3'		
Bonamia ostreae 18S-ITS 1	C_F	5'-CGG-GGG-CAT-AAT-TCA-GGA-AC-3'	760	(Carnegie *et al.*, 2000)
	C_R	5'-CCA-TCT-GCT-GGA-GAC-ACA-G-3'		

Polymerase chain reaction (PCR) amplifications were all conducted in a final volume of 25 μl, consisting of 12.5 μl 2 x DreamTaq™ PCR Master Mix (Thermo Fisher Scientific Inc.) or 12.5 μl 2 x DreamTaq Green PCR Master Mix (Thermo Fisher Scientific Inc.), 0.2 μM forward and reverse primers (Invitrogen), 1 - 5 μl extracted total DNA (20 - 200 ng), and 6.5 - 10.5 μl molecular H_2O (Thermo Fisher Scientific Inc.)

A blank sample containing either 12.5 μl 2 x DreamTaq™ PCR Master Mix (Thermo Fisher Scientific Inc.) or 12.5 μl 2 x DreamTaq Green PCR Master Mix (Thermo Fisher Scientific Inc.), 0.2 μM forward and reverse primers (Invitrogen) and 11.5 μl molecular H_2O was performed with each set of samples as a negative control.

The PCR program ran in a G-STORM 482 - 48 Well Multi Block Thermal Cycler (Gene Technologies Ltd., England) under the conditions in Table 2.2.

Table 2.2. Polymerase chain reaction conditions for the Oe fw_1 + Oe rev_4 primer pair used to amplify *Ostrea edulis* DNA, BO + BOAS, C_F + C_R and BOSTRE-F + BOSTRE-R primer pairs used to amplify *Bonamia ostreae* DNA.

		35 cycles			
Primer pair	**Initial denaturation**	**Denaturation**	**Annealing**	**Extension**	**Final extension**
Oe fw_1 Oe rev_4	5 min 94 °C	60 s 94 °C	60s 45°C	60s 72 °C	10 min 72 °C
BO BOAS	5 min 94 °C	60 s 94 °C	60s 55°C	60s 72 °C	10 min 72 °C
C_F C_R	5 min 94 °C	60 s 94 °C	60s 55°C	60s 72 °C	10 min 72 °C
BOSTRE-F BOSTRE-R	2 min 94 °C	30 s 94 °C	45s 55°C	60s 72 °C	1 min 72 °C

PCR products from the Oe fw_1 + Oe rev_4 and C_F + C_R primer pairs were loaded onto 1 % agarose (Fisher Scientific, UK) gels composed of 100 ml 1X Tris-acetate-EDTA (TAE) buffer and 4 µl ethidium bromide (Sigma-Aldrich®). A 1 kb DNA ladder (Thermo Fisher Scientific Inc.) was used as a reference for the Oe fw_1 + Oe rev_4 primer pair products, whilst a 100 bp DNA ladder (New England Biolabs®Inc., USA) was used as a reference for the C_F + C_R primer pair products.

PCR products from the BO + BOAS and BOSTRE-F + BOSTRE-R primer pairs were loaded onto 2 % agarose (Fisher Scientific, UK) gels composed of 100 ml 1X Tris-acetate-EDTA (TAE) buffer and 4 µl ethidium bromide (Sigma-Aldrich®). A 100 bp DNA ladder (New England Biolabs® Inc., USA) was used as a reference.

Electrophoresis was conducted at 100 V for 1 h, following this the samples were visualised by ultraviolet (UV) transillumination in a 'VWR® Gel Documentation Smart Version system'.

The oysters collected from Langstone Harbour (n = 145) were analysed, for the presence or absence of *B. ostreae*, by the Centre for Environment Fisheries and Aquaculture Science (CEFAS) with all individuals screened using traditional histological methods (OIE 2003). Any samples that showed evidence of infection were confirmed using single round PCR with BO + BOAS and *Bonamia* duplex primers. Any positive products detected were sequenced for confirmation.

2.2.5. Data analysis

Unless stated all statistical analysis was performed in IBM® SPSS® Statistics 25 (IBM Analytics). Benthic survey data collected in 2017 to assess limpet densities within the harbours were analysed using a general linear model (GLM) with harbour and site as independent variables. Oyster data were not suitable for statistical analysis within the GLM due to extremely low numbers across all sampling sites. The mean densities of oysters and limpets within each individual harbour were compared against one another using paired student's T-tests, as were the Chichester Harbour 1998 data. For comparisons between 1998 and 2017 surveys, data were analysed for each species using a GLM with harbour and site as independent variables. A Kruskal-Wallis H test was used to test for differences in limpet chain length and biomass between harbours owing to a non-normal distribution of the dataset. Morphometric data (depth, width, length and weight) were analysed for each separate parameter using one-way ANOVAs against year and site groups. Condition index data were tested for homogeneity of variance using Levene's test and were found to be 'normal' ($F_{1, 46} = 0.9$, $p > 0.05$) and analysed using a one-way ANOVA against location.

FAO-ICALARM stock assessment tools II (FiSat II) modal progression analysis of oyster length frequency distributions were used to identify distinct cohorts within each population. Minimum size class was specified at 15 mm with 5 mm size class intervals. Bhattacharya's method was used to determine initial decompose composite length-frequency distributions and refined using NORMSEP in FiSat II.

2.3. Results

2.3.1 Current abundances of *Ostrea edulis* and *Crepidula fornicata*

During the survey, no oysters were found in Portsmouth Harbour, two individuals were sampled at two separate locations within Langstone Harbour and one individual was observed within Chichester Harbour, giving total harbour values of 0.0 ±0.0, 0.2 ±0.2 and 0.1 ±0.1 oysters / m^2 (mean ±SE), respectively (Fig. 2.4).

In contrast, *Crepidula fornicata* was abundant in many sample areas throughout all three harbours. The highest densities recorded were 900 ±375, 926.7 ±487, 4043.3 ±2374.2 limpets / m^2 within Portsmouth, Langstone and Chichester harbours, respectively. *Crepidula fornicata* were present in 13 of 30 locations within Portsmouth Harbour, 26 of 31 locations within Langstone Harbour and 19 of 31 locations within Chichester Harbour. The overall harbour densities were 84.1 ±24.5, 174.3 ±34.5 and 306 ±106 limpets / m^2 for Portsmouth, Langstone and Chichester, respectively (Fig. 2.5). Both Langstone and Chichester harbours contained significantly more individuals than Portsmouth Harbour, there was no statistically significant difference between Langstone and Chichester harbours ($F_{2, 30} = 4.1$, $p \geq 0.05$). Significantly more *C. fornicata*, 189.0 ±39.0 limpets / m^2, were found across all three harbours in comparison to *Ostrea edulis*, 0.1 ±0.1 oysters / m^2 (pair student's T Test, $t = 4.9$, $p \leq 0.001$).

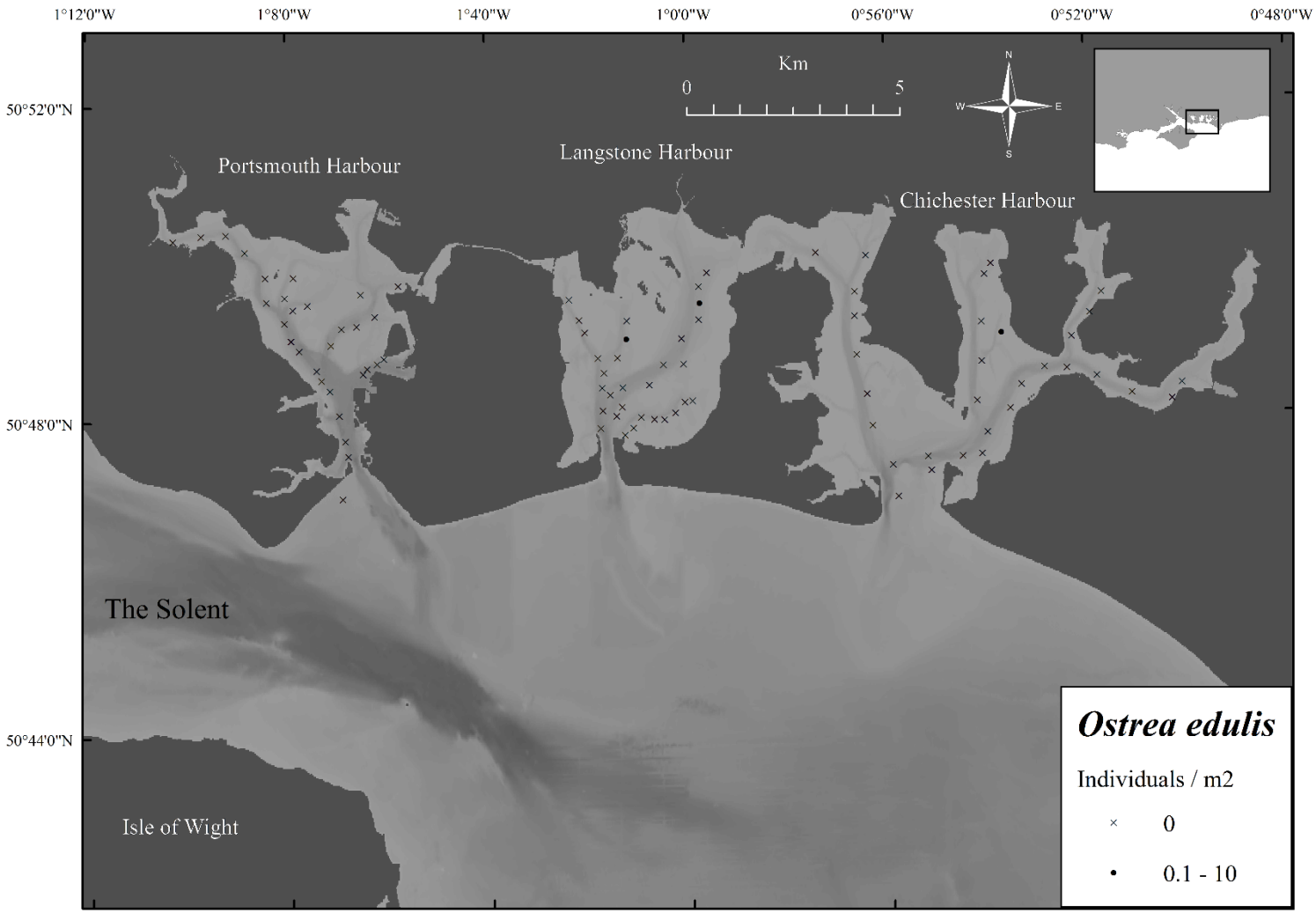


Figure 2.4. Mean densities and distribution of *Ostrea edulis* at the sampling locations in Portsmouth, Langstone and Chichester harbours, 2017. Map created using ArcMap software.

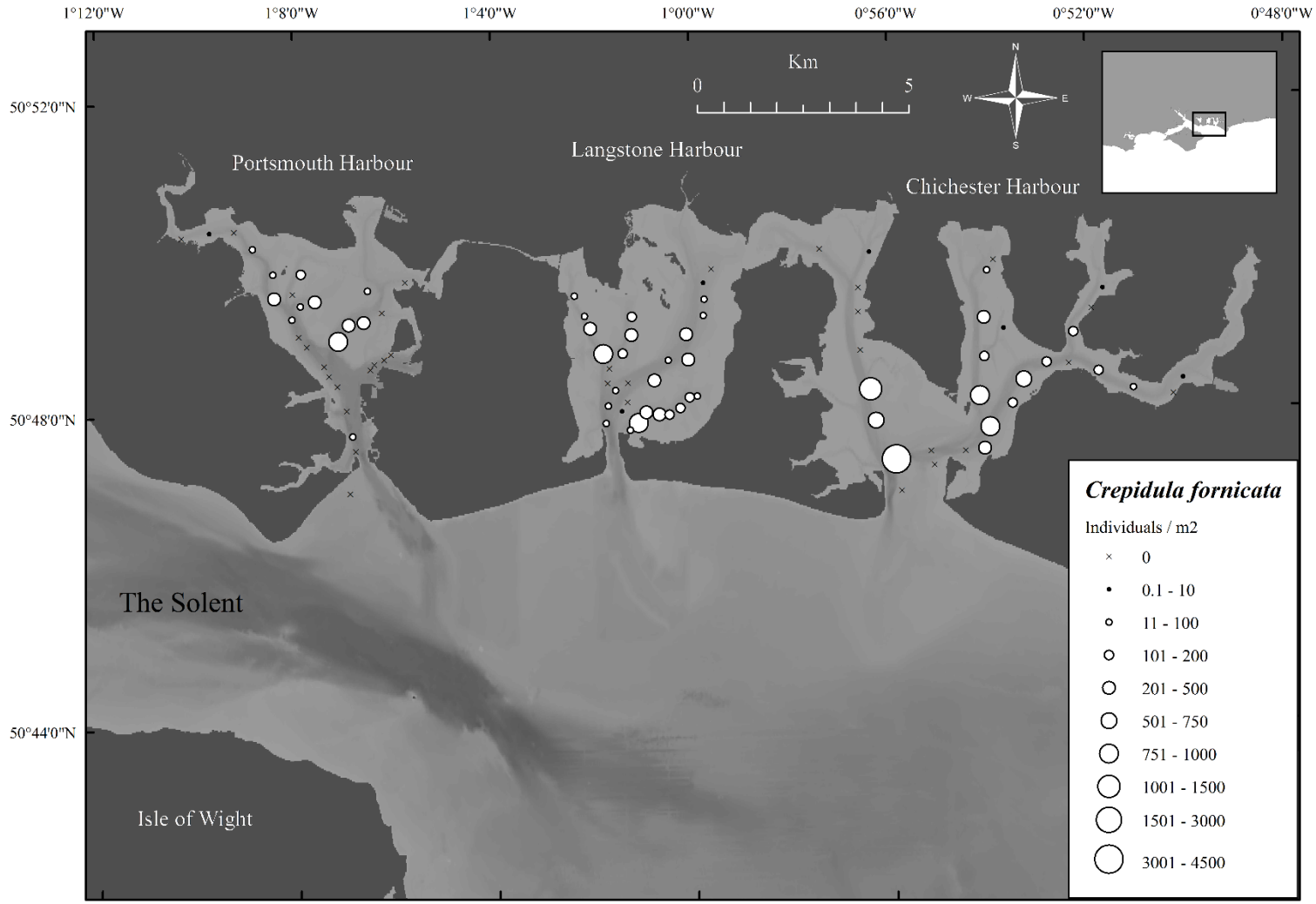


Figure 2.5. Mean densities and distribution of *Crepidula fornicata* at the sampling locations in Portsmouth, Langstone and Chichester harbours, 2017. Map created using ArcMap software.

As well as having the greatest number of limpets / m^2, the longest average chain length of live *C. fornicata* was within Chichester Harbour, 4.7 ±0.2 (mean ±SE), however, this was not significantly longer than those within Portsmouth (4.1 ±0.1) or Langstone (4.0 ±0.2) harbours (Kruskal-Wallis H Test, H = 0.050, p = 0.823 and H = 0.540, p = 0.463, respectively). There was also no significant difference between the chain lengths within Portsmouth and Langstone harbours (H = 0.865, p = 0.352) (Fig. 2.6A).

In contrast to having the shortest average chain length, the weight of each individual limpet chain was greatest within Langstone Harbour, 26.5 ±1.7 g (mean ±SE), significantly heavier than in Portsmouth Harbour, 15.9 ±0.7 g (H = 33.178, $p < 0.001$). The mean chain weight within Chichester Harbour, 23.9 ±0.7g, was also significantly greater than that in Portsmouth Harbour (H = 36.510, $p < 0.001$). There was no statistical difference between chain biomass in Langstone and Chichester harbours (H = 1.630, p = 0.202) (Fig. 2.6B).

The main settlement substratum for *C. fornicata* within all three harbours was found to be dead *C. fornicata* shell with 92.8, 75.6 and 95.5% of chains settled on this substratum within Portsmouth, Langstone and Chichester harbours, respectively (Fig. 2.7). The percentage of live *C. fornicata* at the base of the chain varied across the harbours, with Portsmouth and Chichester having relatively few, 0.9 and 1.4 %, respectively, whilst this occurred with 10.2 % of chains in Langstone Harbour. Very few chains of limpets, < 0.5 %, were attached to oyster shell within each harbour. Attachment to stone accounted for the second highest percentage within all three harbours, 5 % within Portsmouth, 14.2 % in Langstone and 1.4 % in Chichester Harbour. All other attachments accounted for < 1 % in each harbour. Settlement on dead *C. fornicata* shell accounted for 92.2 % within all harbours (pooled data), with attachment to stone accounting for 4.0 %, live *C. fornicata* 2.5 %, oyster shell 0.3 %, cockle shell 0.6 %, whelk shell 0.3 % and periwinkle 0.1 % (Fig. 2.7).

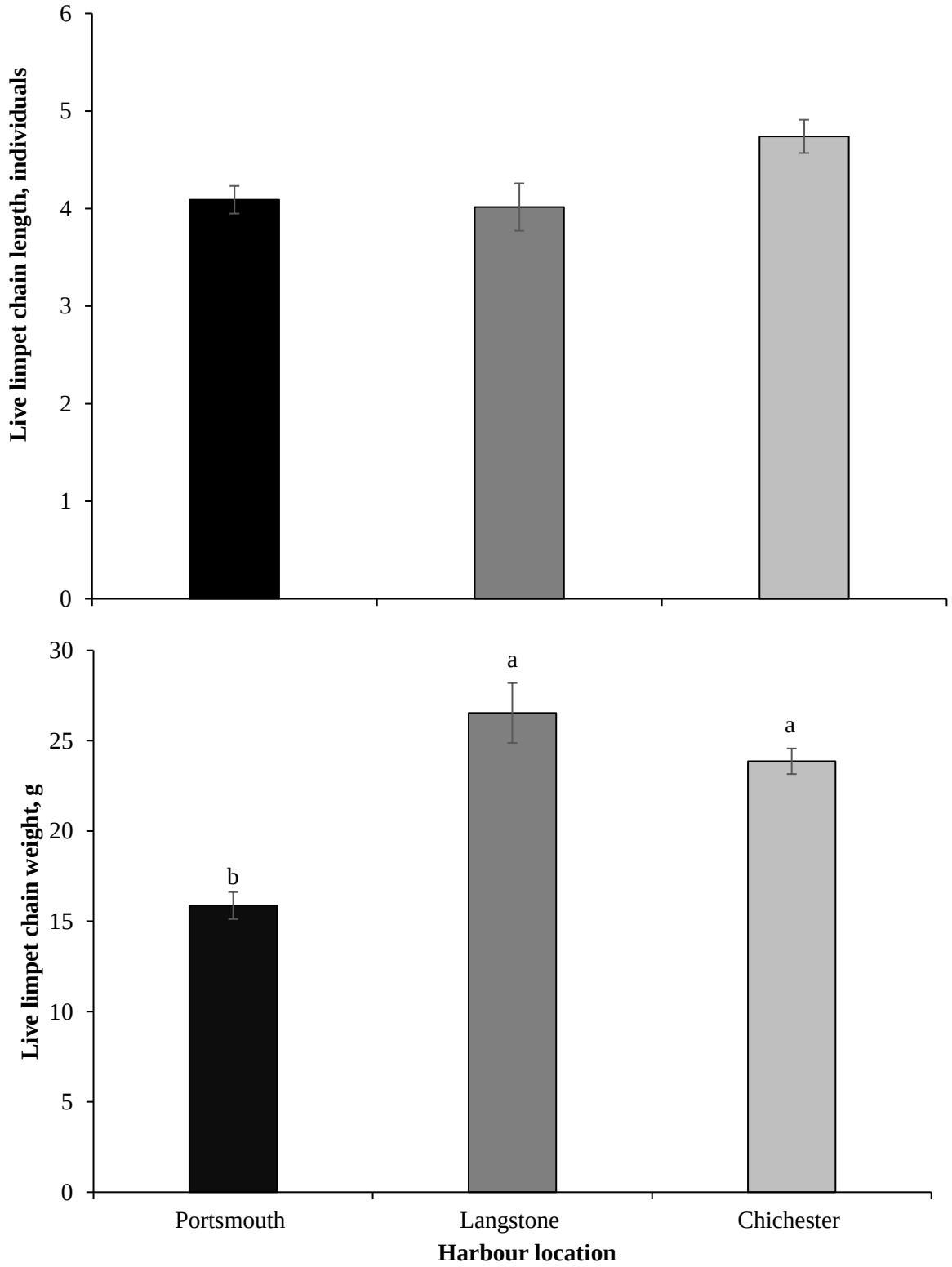


Figure 2.6. Mean (±SE) *Crepidula fornicata* (A) live chain lengths and (B) live chain biomass for the three harbours sampled during 2017, Portsmouth (n = 221), Langstone (n = 127) and Chichester (n = 584). Chain length and biomass was determined for each chain of *C. fornicata* individuals attached to one another in a single mass, irrespective of the direction of attachment and excluding any deceased shells used as attachment substratum. Chains were considered to be separate when the substratum had multiple chains attached to it and these chains were not interconnected by live individuals. Lower-case data labels indicate any significant differences between locations ($p < 0.05$).

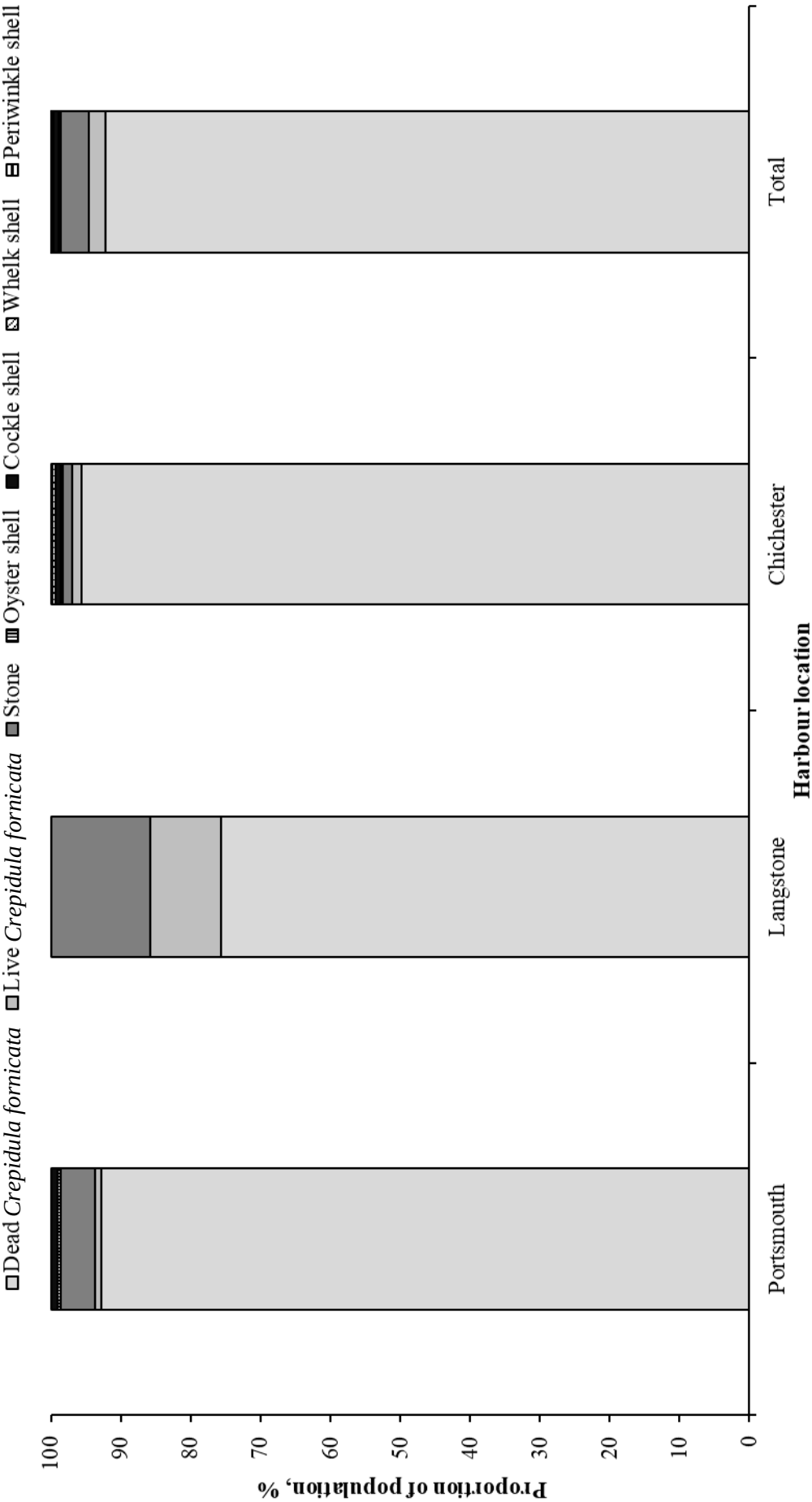


Figure 2.7. Percentage of attachment substrata for live *Crepidula fornicata* chains observed within Portsmouth (n = 221), Langstone (n = 127) and Chichester (n = 584) Harbours, as well as the total for all locations. Each chain of *C. fornicata* was defined as the individuals attached to one another in a single mass, irrespective of the direction of attachment and excluding any deceased shells used as attachment substratum. Chains were considered to be separate when the substratum had multiple chains attached to it and these chains were not interconnected by live individuals.

2.3.2. Long term data comparison of *Ostrea edulis* and *Crepidula fornicata* densities within Chichester Harbour

Ostrea edulis were present in many areas of Chichester Harbour surveyed in 1998, with 14 out of 29 sites providing positive hauls (Farrell, 1998). Mean densities per sample site ranged from 0 to 88 oysters / m^2 and the mean harbour density was 8 ±2.7 oysters / m^2 (mean ±SE) (Fig. 2.8).

Crepidula fornicata was also present in many areas of the harbour in 1998, 19 of the 29 sites. Mean densities per sample site ranged from 0 to 1224 limpets / m^2 and the mean harbour density was 181.2 ±40.7 limpets / m^2 (mean ±SE) (Fig. 2.9). At this period there were significantly more *C. fornicata* than *O. edulis* (T Test, $t = 4.9$, $p \leq 0.001$).

A significant decrease in *O. edulis* density was observed between 1998 and 2017, from 8 ±2.7 to 0.1 ±0.1 oysters / m^2 (mean ±SE) ($F_{1,172} = 19.3$, $p < 0.001$) (Fig. 2.10). In comparison, a significant increase was observed in *C. fornicata* densities between 1998 and 2017, from 181.2 ±40.7 to 306 ±106 limpets / m^2 (mean ±SE) ($F_{1,142} = 10.4$, $p \leq 0.01$) (Fig. 2.11).

Figure 2.8. Mean densities and distribution of *Ostrea edulis* in Chichester Harbour, 1998. Map created using ArcMap software.

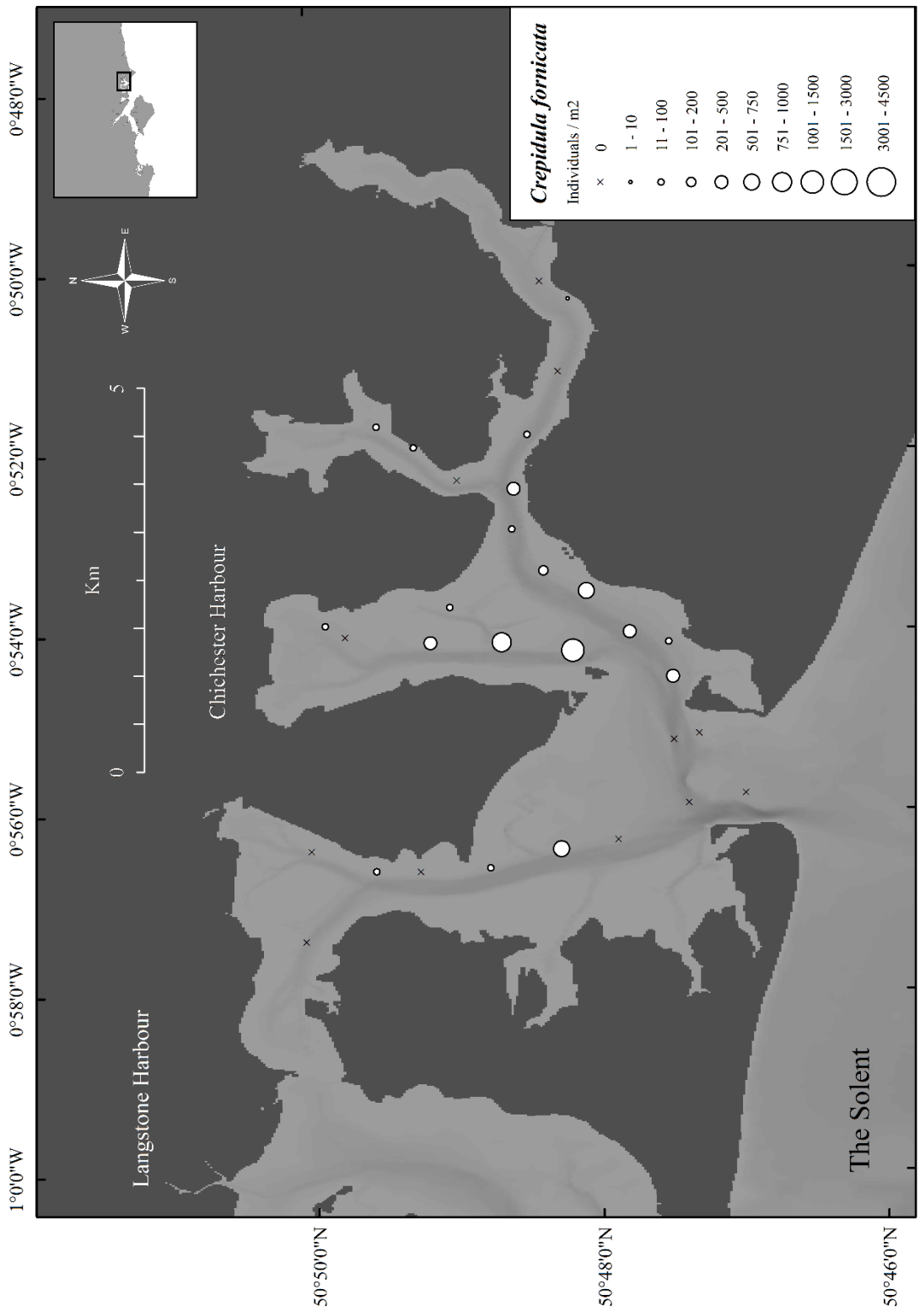


Figure 2.9. Mean densities and distribution of *Crepidula fornicata* in Chichester Harbour, 1998. Map created using ArcMap software.

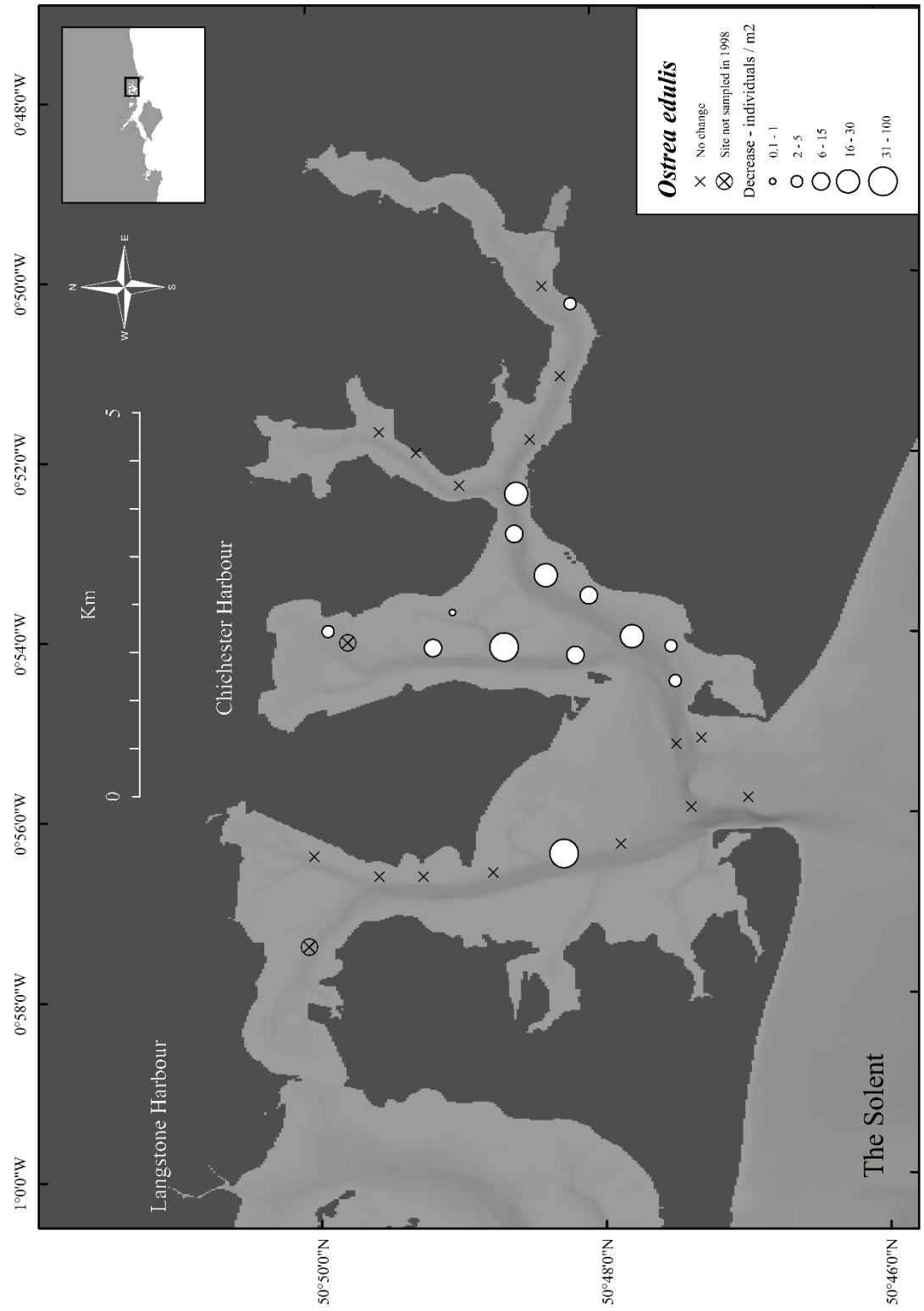


Figure 2.10. Change in *Ostrea edulis* density and distribution in Chichester Harbour from 1998 to 2017. Map created using ArcMap software.

Figure 2.11. Change in *Crepidula fornicata* density and distribution in Chichester Harbour from 1998 to 2017. Map created using ArcMap software.

2.3.3. Population demographics

The Portsmouth 2015 population had a maximum shell length (MSL) of 84.27 ±0.44 mm (mean ±SE), maximum shell width (MSW) of 79 ±0.48 mm, maximum shell depth (MSD) of 33.1 ±0.41 mm and mean weight of 139 ±2.34 g. The Chichester Harbour 2015 population had a MSL of 73.85 ±0.45 mm, MSW of 73.84 ±0.39 mm, MSD of 20.29 ±0.33 mm and mean weight 79.42 ±1.54 g. The Langstone Harbour 2016 population had a MSL of 70.02 ±0.36 mm, MSW of 71.02 ±0.34 mm, MSD of 23.03 ±1.53 mm and mean weight of 85.72 ±1.52 g (mean ±SE). The combined Chichester and Langstone harbours 2017 population had a MSL of 69.96 ±0.38 mm, MSW of 70.88 ±0.36 mm, MSD of 23.13 ±0.24 mm and maximum weight of 87.22 ±2.46 g.

The interquartile range, median and range of the populations are shown in Figure 2.12 A - D with statistically significant populations distinguished by lettering. There were statistically significant differences between group means across site / years (Table 2.3). There was a significant difference in the mean maximum length, width, depth and weight between the oyster populations in Portsmouth and Chichester harbours in 2015. There was no significant difference in mean width or depth between the 2015 Chichester and 2016 Langstone populations. Mean length and width have decreased across sites and years since 2015; the 2017 Chichester and Langstone population has significantly smaller oysters than all previous year / site groups.

The most frequent length size class recorded from the Portsmouth 2015 population was 81 - 90 mm (36 %) in contrast to 61-70 mm (33.86 %) in the Chichester population. The latter was also the most frequent size class in the Langstone 2016 (40.57 %) and Chichester & Langstone 2017 samples (40.69 %). The most frequent maximum shell width size class was 71 - 80 mm in Portsmouth 2015 (30.43 %) Chichester 2015 (30.43 %) and Langstone 2016 (37 %) populations and 61 - 70 mm in the Langstone & Chichester 2017 (43.85 %)

combined population. The demographic structure is narrower within the 2016 and 2017 populations sampled (Fig. 2.12).

The NORMSEP modal progression analysis used to identify the number of cohorts, or age classes from the size frequency data confirmed the narrowing demographic structure and lack of recruitment cohorts (Table 2.4). The number and distribution of the cohorts suggest low levels of recent recruitment across all harbours and years. Three modes were estimated from the Portsmouth size class frequency data but dominated (n = 652) by the smallest cohort with an estimated mean of 84.57 ±9.67 mm (modal mean ±SD) with a tail of low frequency larger size classes, effectively suggesting a single aged cohort. Three more evenly distributed modes were identified in the 2015 Chichester population with a smaller cohort of 71.73 mm (n = 559). The temporal trend demonstrates a decreasing population structure. Only two cohorts were identified in both the 2016 Langstone and 2017 Langstone & Chichester populations, the latter dominated almost entirely by a single cohort (n = 743/757) of 71.20 ±8.78 mm.

Of the oysters recorded from the Portsmouth, Langstone and Chichester Harbour fisheries 80.3, 90.3 and 65.6 %, respectively, were not observed to have any obvious signs of attachment point around the hinge area. *Crepidula fornicata* shell was found to be the attachment point for 11.3, 8.6 and 30.4 % of the populations within Portsmouth, Langstone and Chichester Harbours, respectively, in all cases attachment was to a deceased slipper limpet with the majority attaching within the outer surface of the shell. Oyster shell was found to be the attachment point for 8.4, 1.1 and 4.0 % of the populations within Portsmouth, Langstone and Chichester Harbours, respectively (Fig. 2.13).

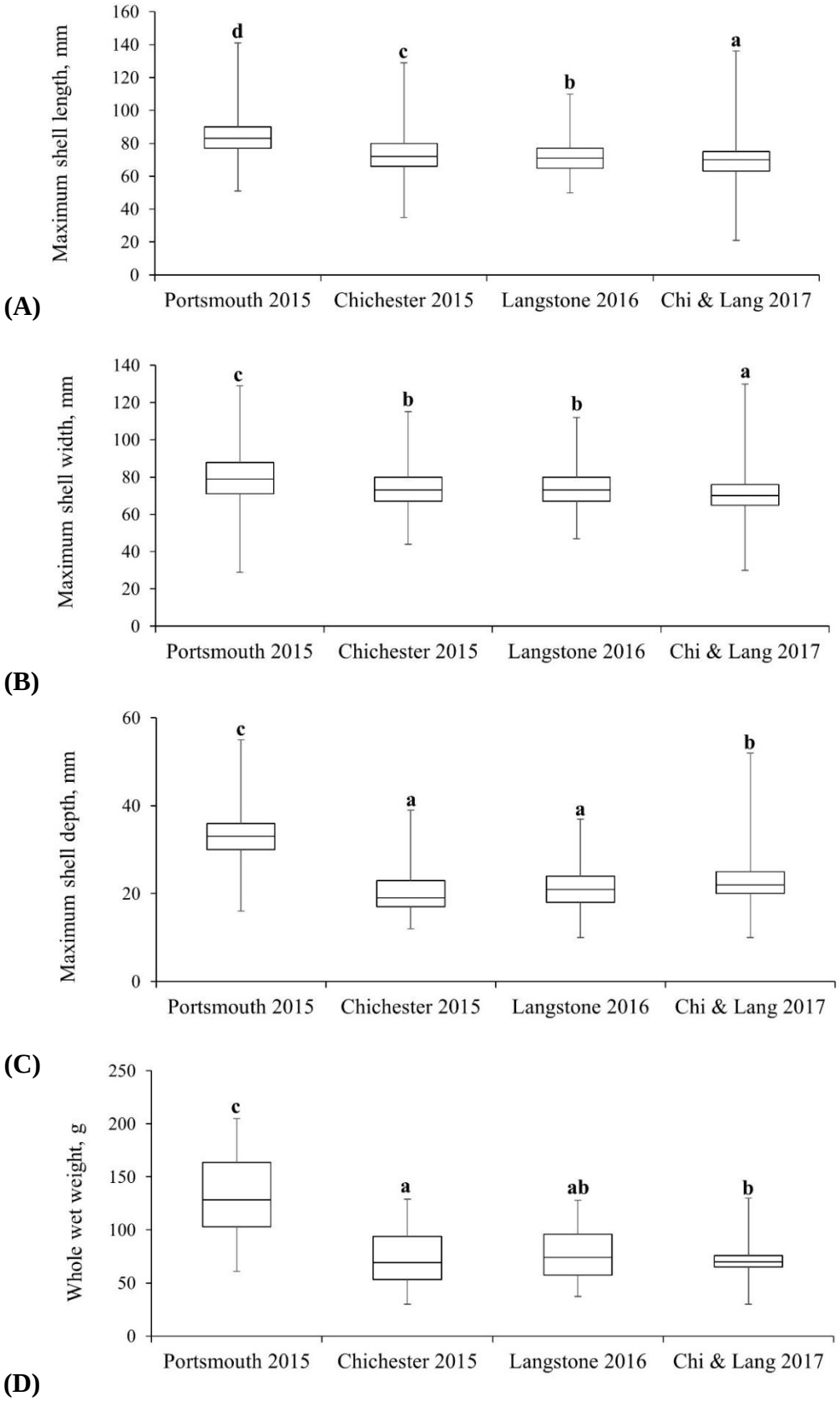


Figure 2.12. Box plots of morphometric parameters (Interquartile range, median and range of maximum (A) length, (B) width, (C) depth, mm and (D) weight, g for *Ostrea edulis* populations (n=700) across eastern Solent harbours during 2015 - 2017, Chi & Lang refers to mixed population from Chichester and Langstone Harbour. Statistical differences are indicated by lettering above the error bars for each group.

Table 2.3. Comparison of fishery population morphometrics from 2015 - 2017.

Morphometrics	Portsmouth 2015	Chichester 2015	Langstone 2016	Langstone& Chichester 2017	Statistical difference between all group means across site/year
Length mm mean ±SE	84.27 ±0.44	73.85 ±0.45	70.02 ±0.36	69.96 ±0.38	$F_{3,2853} = 259, p = \leq 0.001$
Width mm mean ±SE	79 ±0.48	73.84 ±0.39	71.02 ±0.34	70.88 ±0.36	$F_{3,2853} = 89.8, p = \leq 0.001$
Depth mm mean ±SE	33.1 ±0.41	20.29 ±0.33	23.03 ±1.53	23.13 ±0.24	$F_{3,1853} = 305.9, p = \leq 0.001$
Weight g mean ±SE	139 ±2.34	79.42 ±1.54	85.72 ±1.52	87.22 ±2.46	$F_{3,2853} = 329.4, p = \leq 0.001$

Table 2.4. Computed modal mean ± SD length (mm) cohort estimates.

	Portsmouth 2015		Chichester 2015		Langstone 2016		Langstone & Chichester 2017	
Cohort/age class	Mean ±SD	n	Mean ±SD	n	Mean ±SD	n	Mean ±SD	n
1	84.57 ±9.67	652	71.73 ±8.18	559	69.12 ±6.18	400	71.20 ±8.78	743
2	107.62 ±6.26	43	89.35 ±8.66	133	79.08 ±8.76	300	103.33 ±6.53	14
3	126.97 ±2.65	5	124.19 ±6.28	8				

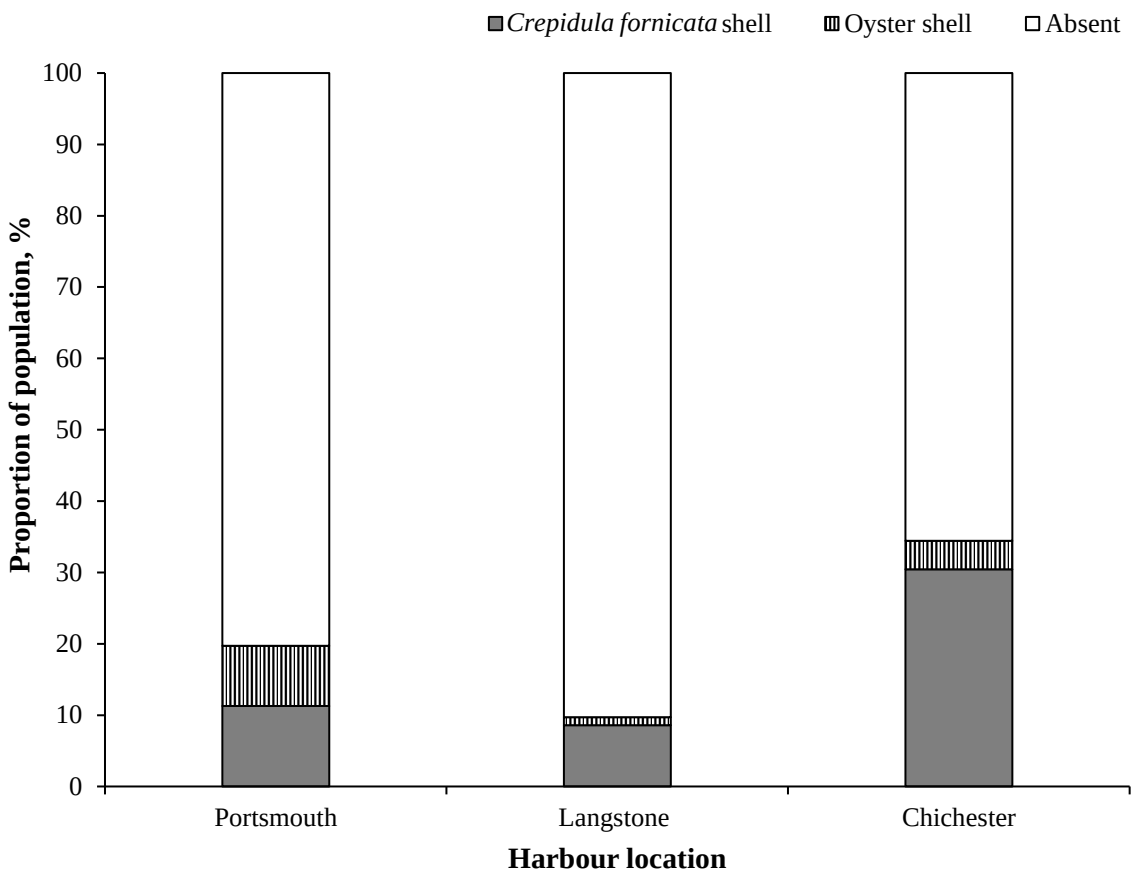


Figure 2.13. Proportion of the oysters (harbour n = 700) retrieved from the fisheries within Portsmouth, Langstone and Chichester Harbours settled to *Crepidula fornicata* shell, oyster shell or with no obvious attachment point observed.

2.3.4. Condition index and occurrence of *Bonamia ostreae*

There was no statistically significant difference between the condition indices of Chichester (3.3 ±0.5 g dry wt (mean ±SE)) and Portsmouth populations (3.97 ±0.5 g dry wt) ($F_{1, 46} = 0.9$, $p > 0.05$). Of the 96 oysters tested, 100 % provided positive amplifications for *O. edulis* at using the Oe fw_1 + Oe rev_4 primer pair. Evidence of *B. ostreae* DNA within Chichester Harbour was detected in 50, 72.9 and 18.8 % of oysters suing the BO + BOAS, BOSTRE-F + BOSTRE-R and C_F + C_R primer pairs, respectively. Evidence of *B. ostreae* DNA within Portsmouth Harbour was detected in 18.8, 25 and 0 % of oysters suing the BO + BOAS, BOSTRE-F + BOSTRE-R and C_F + C_R primer pairs, respectively. A small proportion (4.1 %) of the sample population from Langstone Harbour showed positive products and were sequenced, showing 100 % identity to *B. ostreae* (KY296102.1) with those infected showing light to moderate levels (CEFAS, pers. comm.). Examples of the resulting electrophoresis gels are shown in Figure 2.14.

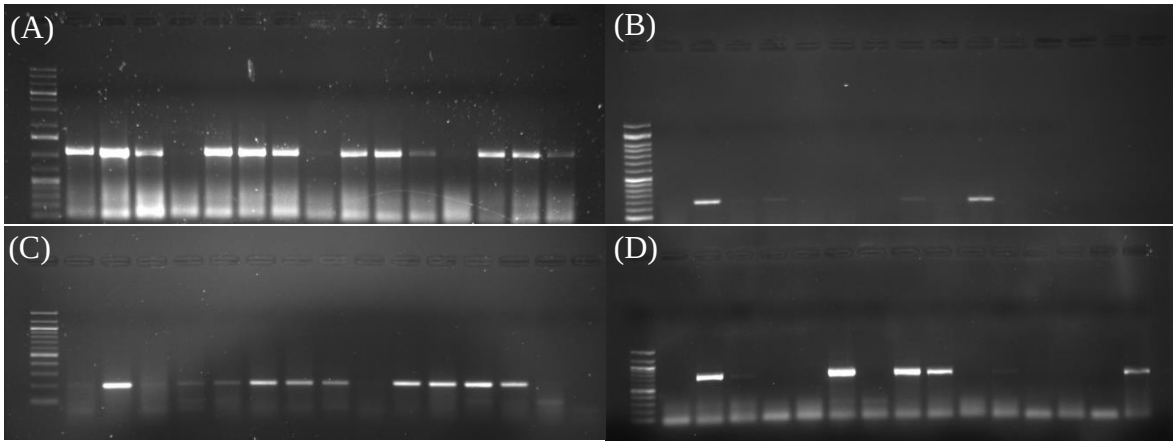


Figure 2.14. Electrophoresis gels under UV transillumination. Left most lane showing 100bp or 1Kb ladders of known fragment lengths. Positive amplififcations are shown by white bands at expected bp values for (A) *Ostrea edulis* cytochrome c oxidase I gene at 1,100 bp using Oe fw_1 + Oe rev_4 primer pair (Gercken & Schmidt, 2014), (B) *Bonamia ostreae* SSU at 300 bp using the BO + BOAS primer pair (Cochennec *et al.*, 2000), (C) *B. ostreae* 18S-ITS1 rRNA gene region at 208 bp using the BOSTRE primer pair (*Ramilo et al.*, 2013) and (D) *B. ostreae* 18S gene at 760 bp using the C_F + C_R primer pair (Carnegie *et al.*, 2000).

2.4. Discussion

In addition to the comprehensive stock assessment conducted by the Southern IFCA (Southern IFCA, 2017, 2018b) these data presented are essential for determining the relative distribution and benthic composition of oysters and limpets to provide a baseline status of the eastern Solent. The nature of the benthic sampling methodology employed for the current study is limited in the area effectively sampled but, in combination with IFCA survey data, demonstrates the disparity between oyster and slipper limet densities. The information provided can be used to inform restoration initiatives and determine the future success of restoration activities proposed in the Solent (e.g. Harding *et al*., 2016). The narrowing of fishery landing sizes combined with the significant decrease in the abundance of the ecosystem engineer, *Ostrea edulis*, within Chichester, Langstone and Portsmouth harbours indicated that these populations of native oysters are not recovering, recruiting or present in reproductively relevant densities.

The long-term decline of *O. edulis* standing stock is caused by a combination of chronic overfishing, poor water quality (Environment Agency, 2016) and disease, which suggests a loss of the ecosystem services that biogenic oyster habitats can provide. As demonstrated by studies of various oyster species, such a loss will likely have a profound negative impact on biodiversity, benthic community structure, trophic pathways and water quality across the Solent as it is highly probable that oysters and oyster reefs are universal in terms of ecosystem services provision (Lenihan, 1999; Jackson *et al.*, 2001; Peterson *et al*., 2003; Tolley & Volety, 2005; Smyth & Roberts, 2010). The continued expansion of the invasive, non-native and highly successful gastropod, *Crepidula fornicata*, has been facilitated by the decline in oysters, impoverished habitat, global shipping movements and a prolific lifecycle (Richard *et al.*, 2006). The presence of such high densities highlights the

impoverished state of the habitat, now a silty mud-dominated benthos which presents a barrier to the restoration of oyster-related benefits that previously once existed (Korringa, 1946; Barnes *et al*., 1973; Erhold *et al.*, 1998; Thouzeau *et al*., 2003; Streftaris & Zenetos, 2006).

First sighted in oyster ponds in Bosham, 'Portsmouth Bay', during 1913 (Cole, 1952), and later in the wider Solent in 1930, *C. fornicata* spread west during the 1940s (Blanchard, 1997). By the 1970s the Solent was almost characterised by *C. fornicata* dominated associated macrofauna. However, *O. edulis* still occurred with a 19 % frequency, which was greater in the West (45 %) than the East Solent (9 %), (Barnes *et al*., 1973). This persistent and increasing dominance of *C. fornicata* since the 1970s is of serious concern for the natural recovery of *O. edulis,* particularly the extremely high densities of over 4000 m^2 within Chichester Harbour which demonstrates the ecological carrying capacity of inshore waters for this invasive species.

The lower densities of limpets within Portsmouth, relative to the other harbours, is likely to reflect the impact of the capital dredge activity that took place to accommodate the arrival of the aircraft carrier HMS Queen Elizabeth (R08) into the naval base (Hopper, 2017). It is evident that *C. fornicata* is absent from all but one of the sampling locations within the main channel that was subject to dredge activity and that the species is still abundant in the central and northern areas of the harbour where dredging did not take place. It is unlikely that many species will continue to inhabit this area due to the historic and ongoing maintenance and capital dredge programs for the area and therefore, it should be completely disregarded from any benthic restoration activities. In addition, the reduced biomass of the chains indicates that the individuals that form these chains are of a reduced size in comparison to the other harbours and that, despite being abundant in some areas, overall this harbour provides a less suitable habitat for the species. It is evident that the environmental

conditions currently present within Langstone Harbour are facilitating the continued dominance of *C. fornicata* and that the species has become wide spread, densely populated with multiple year classes present and, despite having slightly fewer individuals per chain than the other harbours these individuals have a significantly greater biomass. The increased chain length compared to chain biomass within Chichester Harbour is likely to be reflective of recent recruitment to the area with many smaller individuals contributing to the length of each chain but therefore reducing the biomass of such chains.

The preference of *C. fornicata* to settle on the shells of deceased individuals of the same species poses extremely difficult logistical issues during the decision-making process surrounding the seabed management of areas intended for the restoration of *O. edulis* when such high densities of *C. fornicata* are present. The attachment material of those oysters observed within the fishery also reflects the current abundances of live *C. fornicata* and *O. edulis* populations and supports the findings that the settlement of oyster larvae is limited by the availability of suitable substrata, not the nature of the available substrata (Smyth *et al.*, 2018). This study demonstrates that the shell of deceased *C. fornicata* can be utilised by *O. edulis* for settlement, however, even with extremely dense populations of slipper limpet only a small proportion of oysters utilised this substratum in Portsmouth and Langstone Harbours, with a reasonable proportion doing so in Chichester Harbour. The absence of attachment material for many of the oysters sampled is likely to indicate settlement to stones or rocks as they are easily broken off, as was seen with some individuals not used in this study.

Managing the substrata will be one of the defining practices for successful restoration, with *O. edulis* larvae displaying gregarious behaviour, preferentially settling on conspecifics and hard substrata with a rough, but clean, surface (Cole and Knight Jones, 1939, 1949; Walne, 1964; Bayne, 1969; Walne, 1974). The current state of the benthic habitat will not

accommodate settlement of large quantities of oyster larvae and must be addressed to resolve the issues surrounding the muddy substratum, dominated by high densities of *Crepidula*.

Removal of slipper limpet populations, via dredging, would be costly and time consuming if conducted simply for that purpose. The process may become feasible if the process was commercially viable with an end product that could be utilised for human, livestock or aquaculture consumption, as well as commercial aggregates, however, further research and streamlining would be required for this to occur (Fitzgerald, 2007). Even if this option was applicable, the attempted removal of the limpet populations, to allow the deposition of native oyster populations, is unlikely to extract all individuals and may succumb to a re-invasion as the beds would be unsuitable for continuous dredging due to the necessity for oyster population growth.

Alternative options would involve harrowing of beds to expose clean shell for oyster larvae to settle on. This method has been encouraged for the maintenance or restoration of oyster beds by Abbe (1988) and has been implemented in the Blackwater fishery, Essex (Fowler 1893). Again, if this method was used for areas with large populations of *C. fornicata* then this would expose limpet shell and likely encourage the settlement of more *C. fornicata* as they express strong gregarious behaviour and a preference for this settlement substratum, as seen during this study. The process would need be conducted at an appropriate time prior to the peak in the oyster spawning season in order to allow for the flesh of the limpets to be consumed in time for the clean internal shell to be exposed. There are few examples of harrowing as a management method and those that do exist, show that harrowing is valueless (Waugh, 1972) and in one case that *O. edulis* spat settlement was greater in unharrowed areas (Bromley *et al.*, 2016b). This is also likely to be the case for many of the areas within the Solent as despite having an active fishery, which would have had a similar impact to harrowing, the populations of limpets have persisted and increased

whilst the populations and recruitment of oysters have continued to decline. This method is also limited by the larval densities present to settle on the newly exposed shell, in the case of the Solent this is sporadic and unpredictable.

The complete smothering of the limpet populations, to raise the potential oyster reef area, with a combination of hatchery reared spat on shell and suitable cultch for oyster settlement such as oyster shell, scallop shell or locally sourced stone, is another option that should be considered for future restoration efforts. Again, an option that is likely to be time consuming and costly, but will allow for the improvements of habitat, oyster density and survival (Colden *et al.*, 2017).

The diet of *C. fornicata* has been shown to be non-selective for particle size and to overlap with that of the oyster species, *Crassostrea gigas* and *O. edulis* (Beninger *et al.*, 2007, Decottignies *et al.*, 2007, Nielsen *et al.*, 2017) with an efficient particle retention (Jørgensen *et al.*, 1984) superior to that of *C. gigas* (Barillé *et al.*, 2006). The high densities of mature *C. fornicata* in the Solent is likely to exert extreme competition stress on *O. edulis* for both habitat and food resources. In future studies the extent to which *C. fornicata*, at high densities, competitively excludes *O. edulis* should be tested.

The presence of *C. fornicata* not only negatively impacts the *O. edulis* broodstock population, but the oyster larvae are also be subject to substantial predation from mature limpets and competition for food and space from their larvae (Korringa, 1951a in Pechenik *et al.*, 2004; Fitzgerald, 2007). *Crepidula fornicata* aggregations form a cohesive calcareous shell-mud in areas where they have become the dominant species, in contrast, hard, clean substrata is preferred by oyster larvae (Smyth *et al.*, 2018). The reduced availability of suitable settlement substrata, due to both the levels of mucus pseudofaeces (Blanchard, 1997) generated by such high densities of *C. fornicata* and the lack of conspecific shell substrate, is a major concern for *O. edulis* larval settlement in restoration areas. A reduction

in preferential substrata will be further compounded by competition for food arising during the overlapping breeding periods of both species, with *C. fornicata* spawning two to four times between February and September (Richard *et al.*, 2006) and *O. edulis* typically spawning once between May and August (Hayward *et al.*, 1996). The reproductive cycle of *O. edulis* is also relatively complex in relation to other oyster species and that of *C. fornicata* which is sexually mature within 2 months (Richard *et al.*, 2006). In comparison, *O. edulis* is not usually mature until individuals reach 3 years old (Roberts *et al.*, 2010). Again, this is problematic, as the remaining natural populations of mature oysters within the sampled harbours of the Solent have decreased significantly in size and abundance over a short time period. This smaller, less mature and narrowed demographic population will negatively impact spawning potential. For example, a decrease in mean size from 80 mm to 70 mm, similar to the decline observed from 2015 - 2017, would result in a reduced output of 260,000 larvae per reproductive female (Walne, 1974). When applied to a fishery, characterised by a skewed sex ratio (6 : 1 male : female sex ratio (Kamphausen *et al.*, 2011)), reproductive and recruitment success will be severely impacted. This is of great concern and as a conservative estimate, 85 % of the 2017 Langstone & Chichester population will be above the minimum landing size for the 2018 / 19 fishing season and therefore at high risk of being extracted to the point of functional extinction (Beck *et al.*, 2011). To put this in context, in 1973 only 22 % of *O. edulis* population in Stanswood Bay were of marketable size > 70 mm (Barnes *et al.*, 1973), and it was this population that was thought to feed the boom of the Solent fishery in the late 1970s and 1980s. The data presented are supported by that of the Southern IFCA (Southern IFCA, 2018) in showing that the majority of the fishery population that is present or that was removed is above the minimum landing size and the limited size classes indicates a lack of recurrent recruitment. This risk of extirpation at current fishing levels seems

particularly high as this removal of mature oysters will also remove the supply of larvae critical to the future of such populations.

The last successful recruitment, estimated from size (Richardson *et al.*, 1993), was approximately six to seven years ago in 2012 for the Langstone & Chichester Populations. The smallest 2015 Portsmouth cohort with a mean maximum length of 84 mm suggests this is the aged remnant population from a successful spatfall eight to ten years ago, approximately in 2008. The morphometric data reveals a disjunct population structure over microgeographic scales within the Solent, particularly between Portsmouth and Chichester Harbours in 2015. This could be attributed to the re-stocking that took place as part of a small-scale restoration project in Chichester during November 2010 (Vause, 2010; Eagling, 2012 cited in Gravestock *et al.*, 2014; MEDIN, 2016), which aligns with the estimate from the demographic cohorts. However, it is concerning that the demographic data showed a lack of recruitment to the seabed in all three harbours despite the previous two years of fishery closure and a reduced fishing season since 2015.

When planning and managing projects to restore such populations, disease control is of utmost importance. The prevalence of *Bonamia ostreae* within the Chichester population in this study has increased from previous years (1993 - 2007 average, 12.1 % (Laing *et al.*, 2014)) and exceed values of Eagling (2012, cited in Gravestock *et al.*, 2014), who reported disease prevalence of 25 - 35 % a few years prior to this. This increase could be attributable to the mortality of many of the re-laid oysters (at a density of 20 / m^2) within the harbour observed by Jensen (pers. comm. with Gravestock *et al.*, 2014) and indicate that this area may be susceptible to future outbreaks of bonamiosis.

In contrast, the parasite was found at a lower prevalence within the Portsmouth population. Despite this, prevalence again increased from previous recordings (1993 - 2007 average, 5.6 % (Laing *et al.*, 2014)). Unlike the increase within Chichester and Portsmouth

populations, a reduction in prevalence was recorded within the Langstone population (1993 - 2007 average, 9.1 % (Laing *et al.*, 2014)). Due to the varying prevalence within the three interconnected harbours it is not clear if the hydrodynamics of the area allow for the dispersal of the parasite in either an easterly or westerly direction either in the water column or via larval transmission (Flannery *et al.*, 2016). Disease transmission between populations on a small geographic scale should be investigated further.

It is clear that the *C. fornicata* dominated benthos of the Solent harbours is in a poor state and, without significant intervention or disturbance, presents a barrier to the return of the native oyster *O. edulis* along with the biogenic habitat and associated biodiversity it provides.

On a final note, water quality is also of concern: all three harbours are classified as Nitrate Vulnerable Zones (NVZ) and designated as both Sensitive Area (Eutrophic) and Polluted Water (Eutrophic). Portsmouth Harbour is currently hypernutrified in regard to nitrogen levels, Langstone Harbour has improved, but remains elevated along with Chichester Harbour (Environment Agency, 2016b). Alongside the ecosystem services for biodiversity, biogeochemical cycles that are maintained by oysters and their associated epibionts, are highly efficient at nitrogen cycling and removal (Kellogg *et al.*, 2013). Restoration of the native oyster habitat *O. edulis* to these harbours has the potential to exert natural eutrophication control (Officer *et al.*, 1982; Dame, 1996), improve water clarity (Newell, 2004) and decrease suspended sediment. In turn this could facilitate seagrass growth (Newell & Koch, 2004) with the associated multi-trophic benefits of restoring both seagrasses and oyster beds.

2.5. Conclusion

The low standing stock of *Ostrea edulis*, coupled with a benthos dominated by high densities of *Crepidula fornicata*, the presence of *Bonamia ostreae* and continued fishing pressure are significant barriers to self-sustaining native oyster populations within the Solent. The high-density populations of *C. fornicata* are likely to facilitate the continued proliferation of the species due to the highly gregarious nature of settlement presented in the results of this study. This obstruction of *O. edulis* settlement will prevent the formation of substantial populations, leading to a genetic bottleneck which is likely to exacerbate the effects of *B. ostreae* and other stressors. Based on the status of *O. edulis* in the commercially fished areas of the Solent presented here, active management of the seabed is recommended to (1) control the extent and spread of *C. fornicata*, (2) provide suitable settlement substrate for *O. edulis* larval recruitment and (3) establish a protected *O. edulis* broodstock population in all commercially fished Solent harbours, in agreement with Fariñas-Franco *et al.* (2018). This chapter highlights the importance of understanding local population structures and disease prevalence over relatively small geographic scales and reinforces the need for relevant and comprehensive baseline data to underpin *O. edulis* restoration practices. All the results highlight the impoverished state of the native oyster, in what was once a substantial population, thus the need to restore the species to the area.

Chapter 3

The Efficacy of Suspended Broodstock Cages as a Restoration Strategy - Mortality

3.1. Introduction

With the marine environment becoming increasingly exploited, marine spatial planning is a key element to many new developments and should be considered by all types of conservation and restoration projects attempting to conduct ecosystem-based work in congested coastal areas (Douvere, 2008; Katsanevakis *et al.*, 2011). Despite congestion, the development of coastal waters provides a variety of opportunities to work with the many stakeholders involved. One such option that has arisen for a variety of conservation and restoration projects it to utilise existing structures to provide additional habitat and structural resources needed to achieve desired results (Mineur *et al*., 2012, Strain *et al*., 2018; Hall, 2018, 2019; Mayer-Pinto *et al.*, 2019). These existing structures offer a variety of opportunities from increasing biodiversity of a range of native species within an area, to species-specific restoration projects such as this study.

This chapter focuses upon a novel method of restoration aquaculture, using broodstock cages to house oysters in high densities below marina pontoons. It was anticipated that successful reproduction would occur in a high proportion of the population when housed in closer proximity and at a higher density than on the seabed. The efficacy of the system was monitored with parameters including broodstock survival, mortality and condition when suspended beneath marina pontoons across a wide geographical area within the Solent, in relation to varying environmental conditions. Further parameters, such as reproduction and larval production, are covered in subsequent chapters. To develop this system two densities were trialled in the first year and the impact of pressure-washing tested in the second year. All the locations selected are currently classified as Nitrate Vulnerable Zones (NVZ) (Environment Agency 2016a, 2016b) and determining if *Ostrea edulis* can indeed survive in these conditions is vital to the future site selection process.

3.2. Methods

3.2.1. Broodstock cage design, cage density and oyster source

3.2.1.1. Year 1: 2016/17 - 17/18

The design process for this study involved the engineering and project management team at Marina Developments Limited (MDL) Group Ltd, project management team at the Blue Marine Foundation and researchers from the University of Portsmouth. The broodstock cages used in this study were modified from a previous study that housed loose oysters in 55 x 55 x 17 cm, 1 " square Aquamesh® cages (GT Products Europe Ltd) (Helmer, 2016), see Appendix B for specifications. Micro-reef structures (Fig. 3.1A) were incorporated and allowed for individual oysters to be maintained within a given position and density. The individual micro-reef sections were attached with hog rings, which overtime were replaced with cable ties, to form a single unit (Fig. 3.1B - C), four of these units were then combined to form one module (Fig. 3.1D). Three modules were then stacked on top of one another (top, middle and bottom positions) and held in place using a 75 cm length of 40 mm polyvinyl chloride (PVC) tubing (Toolstation Ltd) that passed through the central sections of the micro-reef modules. To prevent the modules sliding off the PVC tubing the bottom module was attached to a 160 mm x 160 mm square gully grid (Toolstation Ltd) used as a base (Fig. 3.1E), two further modules were then placed on top of this base module to complete the arrangement for each cage (Fig. 3.1F). The arrangement allowed for a maximum of 120 oysters to be housed, per cage, in the micro-reef slots and was used as the full-density population for the trial. For the half-density population trial, an oyster was placed in every-other slot of the micro-reef structures (Fig. 3.2). Each of these were then housed within a rectangular 2 " Aquamesh® cage (30 cm x 30 cm x 93 cm) (GT Products Europe Ltd) that was suspended beneath marina pontoons using 50 cm of 40 mm link chain

(Fig. 3.3), see Appendix C for specifications. Each of the sample locations began housing five full-density cages and five half-density cages that were monitored with two full-density and two half-density cages used for stock replenishment in the monitored cages across the trial period.

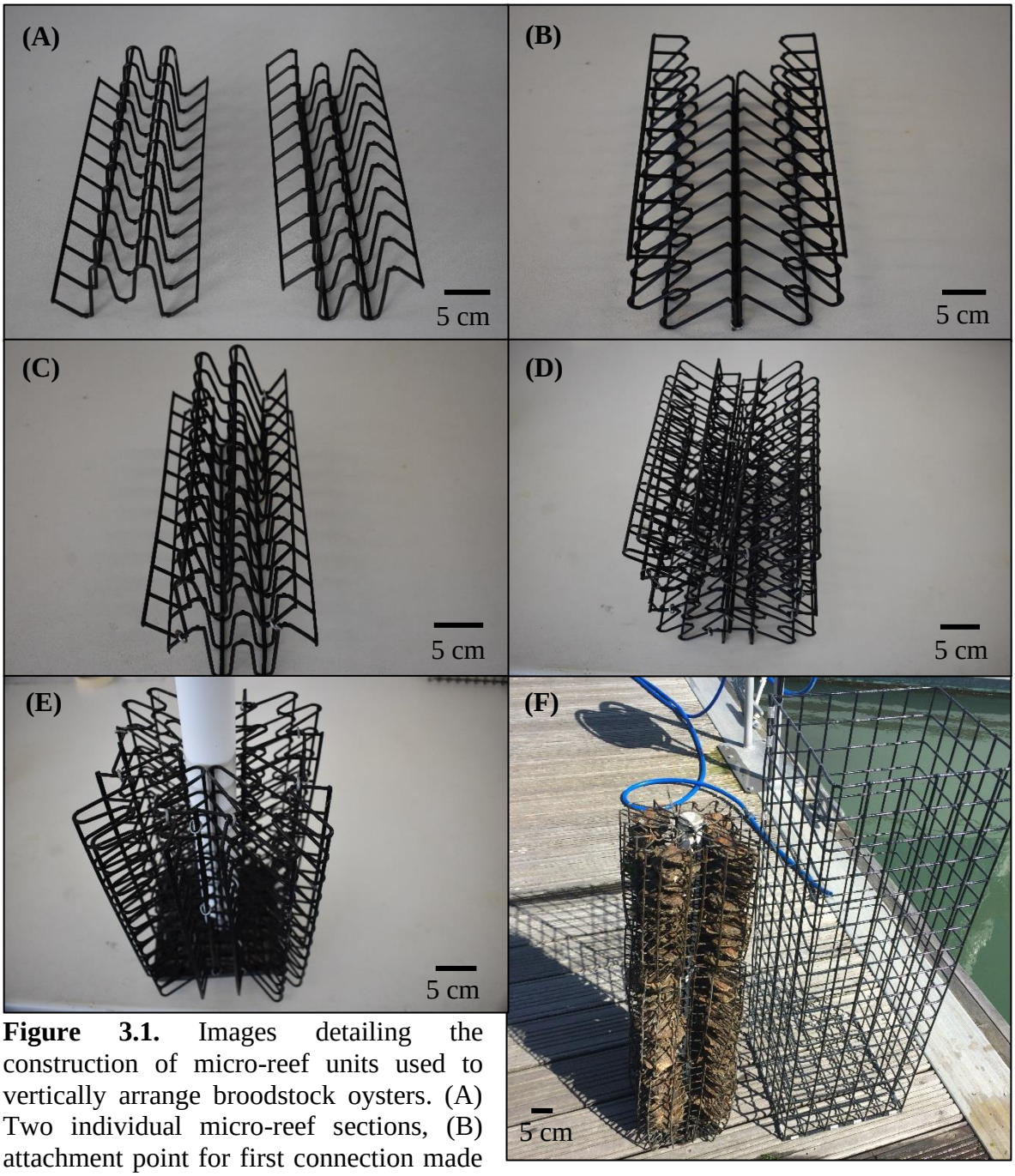
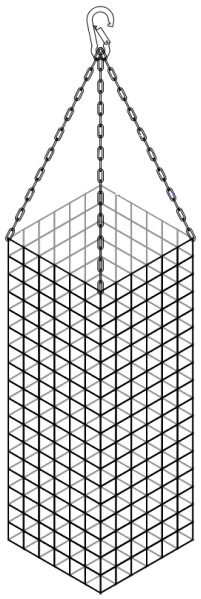


Figure 3.1. Images detailing the construction of micro-reef units used to vertically arrange broodstock oysters. (A) Two individual micro-reef sections, (B) attachment point for first connection made to form a single unit, (C) second attachment point to combine the two sections, (D) four singles units combine to form one module and (E) one module placed over 40 mm polyvinyl chloride tubing with base, (F) two further modules were then placed on top of this base module to complete each cage structure which was then filled with broodstock oysters.



Full-density

Row	Column 1	2	3	4
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				
26				
27				
28				
29				
30				

Half-density

Column 1	2	3	4

Figure 3.2. Schematic of the micro-reef units within the full- and half-density broodstock cages. Empty cells indicate positions where oysters were housed whilst shaded cells indicate positions where oysters were not housed in the half-density population. Bold lines indicate the separation between the top, middle and bottom micro-reef units, each ten rows in height.

Pontoon modifications, to accommodate the cages, at Saxon Wharf, Port Hamble, Hamble Point and Sparkes Marinas were designed and constructed by MDL Marinas Group Ltd and Walcon Marine Ltd. Modifications involved the creation of a hatch from the existing decking on the pontoon walkways. Beneath the pontoon a metal bar was suspended perpendicular to the walkway using existing bolt holes, three snap-hook carabiner clips were then used to attach the cage structures via the chain and suspend with the top of the cage at a depth of 30 cm and the bottom of the cage at a depth of 121 cm (see Appendix C for specifications). Attachment at the Portsmouth and Langstone Harbour locations utilised existing structures to suspend the cages using metal frames that were held in place using grooves (see Appendix B for specifications). All oysters were sourced from the Langstone Harbour fishery during November 2016. Two separate deliveries of approximately 5,000 oysters each were delivered to both Port Hamble Marina and Hamble Point Marina at the beginning of November 2016. Oysters were temporarily suspended in onion bags beneath the pontoons until the beginning of December 2016, they were then placed into micro-reef modules and wrapped in deer fencing prior to the construction of permanent Aquamesh® cages. All oysters remained at Port Hamble and Hamble Point marinas (Fig. 3.4) until March 2017, they were then transferred to the Aquamesh® cages, distributed to the four other sampling locations and acclimatised for two months before monitoring commenced.

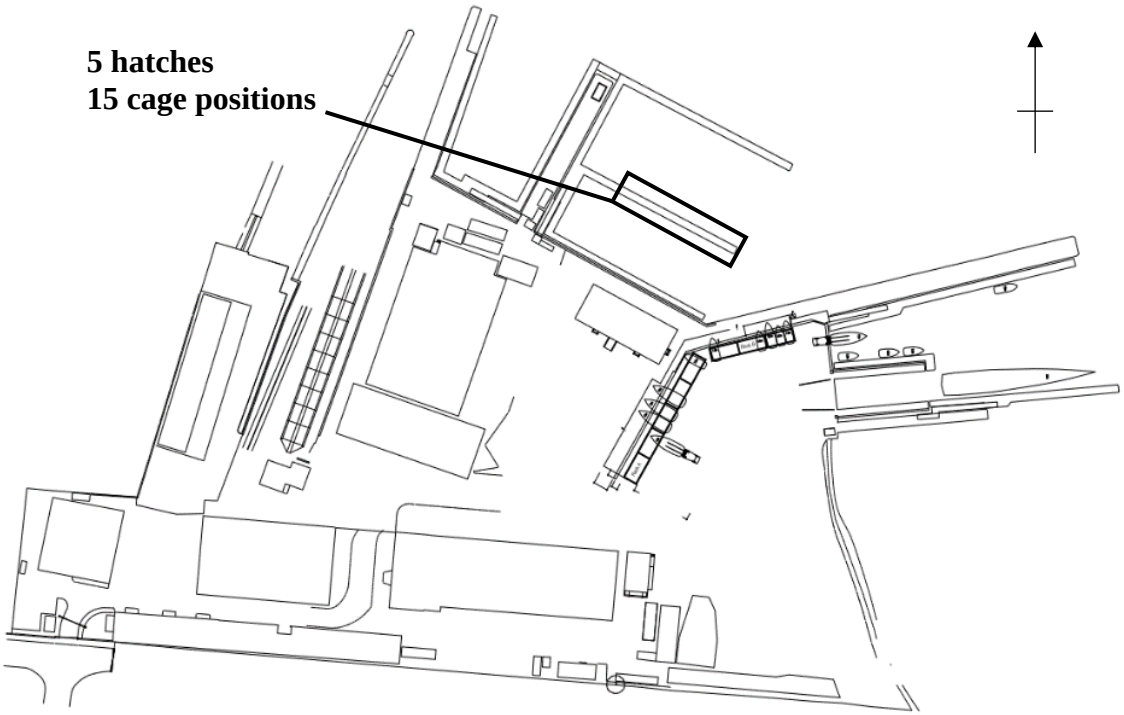
3.2.1.2. Year 2: 2018

The same cages and oysters as in 3.2.1.1 were also used for the second year of treatment and monitoring, which ran from May 2018 to November 2018. Those oysters that remained alive after the first year of treatment conditions in 3.2.1.1 were rearranged into six half-density cages per sampling location with no full-density cages used for the second year's treatment. No stock replenishment took place throughout the 2018 trial.

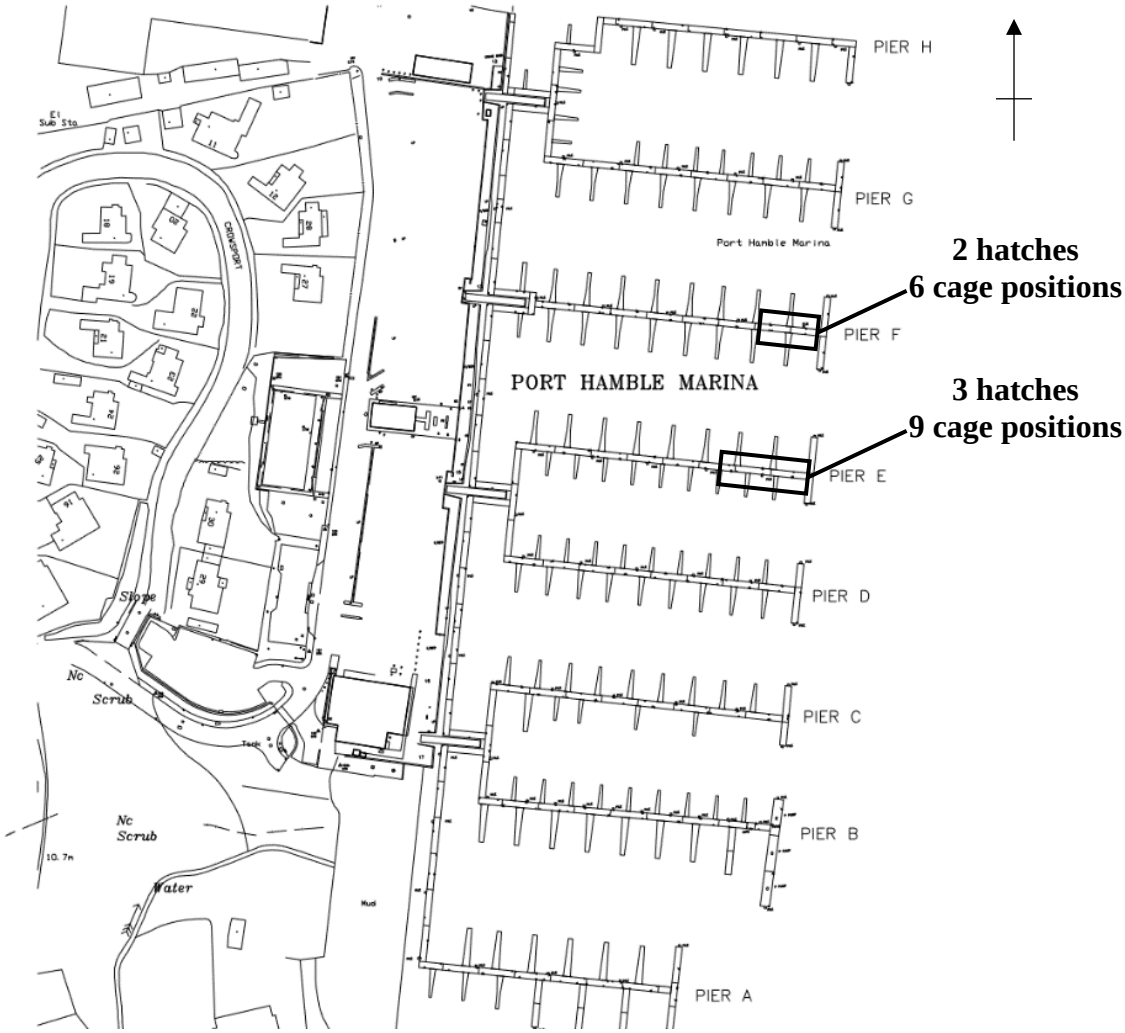
Figure 3.3. Pontoon modifications showing (A) hatch *in situ* on the main pontoon walkway, (B) close up individual hatch unit, (C) Allen key locking mechanism, (D) small opening to allow for the hatch to be lifted up, (E) hatch removed and placed on the pontoon, (F) cages suspended on the metal bar and (G) close up of an individual cage suspended at the surface of the water column. See Appendix C for cage specifications.

3.2.2. Broodstock cage sample locations

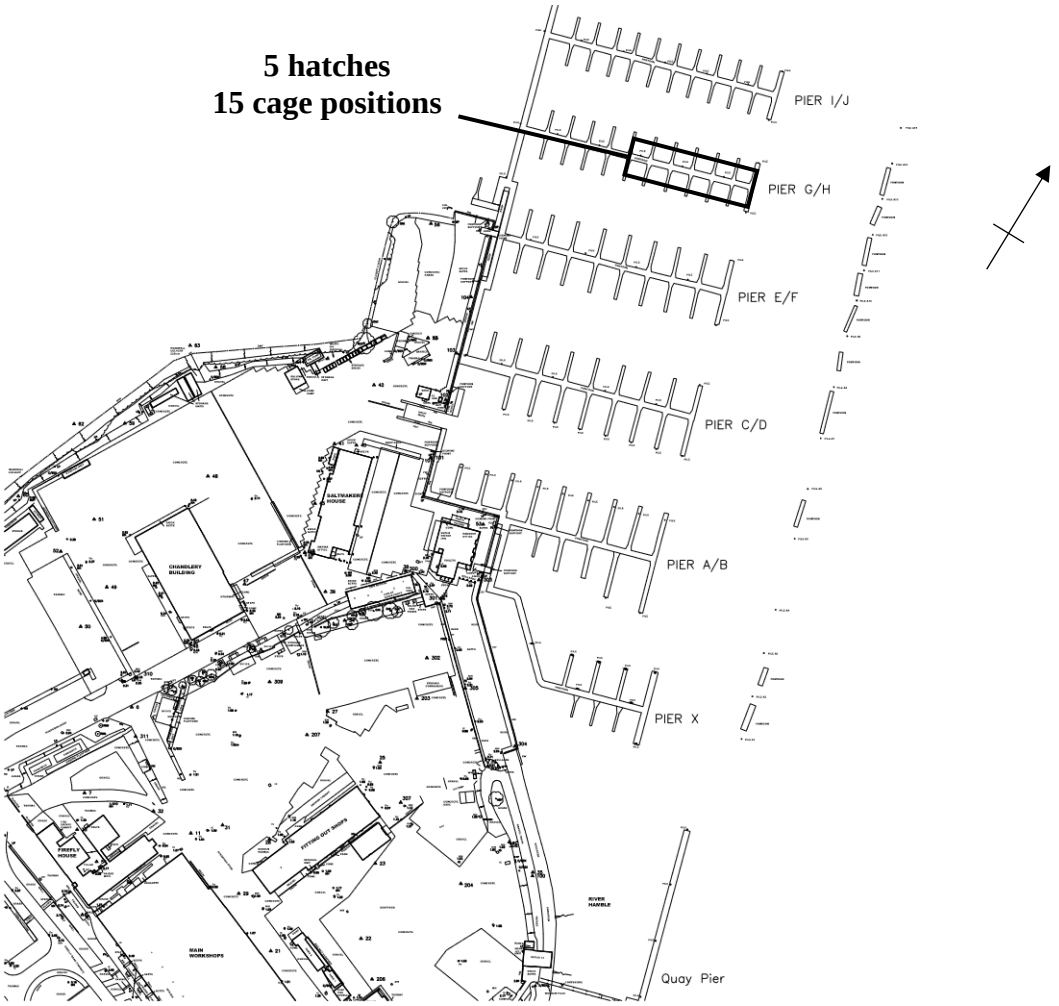
A total of six locations were selected to provide a geographical variation that would reflect as much of the central and eastern Solent as possible, within the remit and scale of the study. The University of Portsmouth research platform (UP) and the research platforms attached to the main pontoon at the INEOS Team UK headquarters (previously Land Rover Ben Ainslie Racing) (BA) at had both been used in the study by Helmer (2016) (Appendices C and E), thus were continued to provide a comparison of cage efficiency. In addition to these sites a further four locations were selected. These included the MDL marina locations of Saxon Wharf (SW), Port Hamble Marina (PH), Hamble Point Marina (HP) and Sparkes Marina (SP) (Fig. 3.4). These sample sites provided a wide range of environmental conditions including sheltered river, exposed river, sheltered harbour and exposed harbour environments (Fig. 3.5).



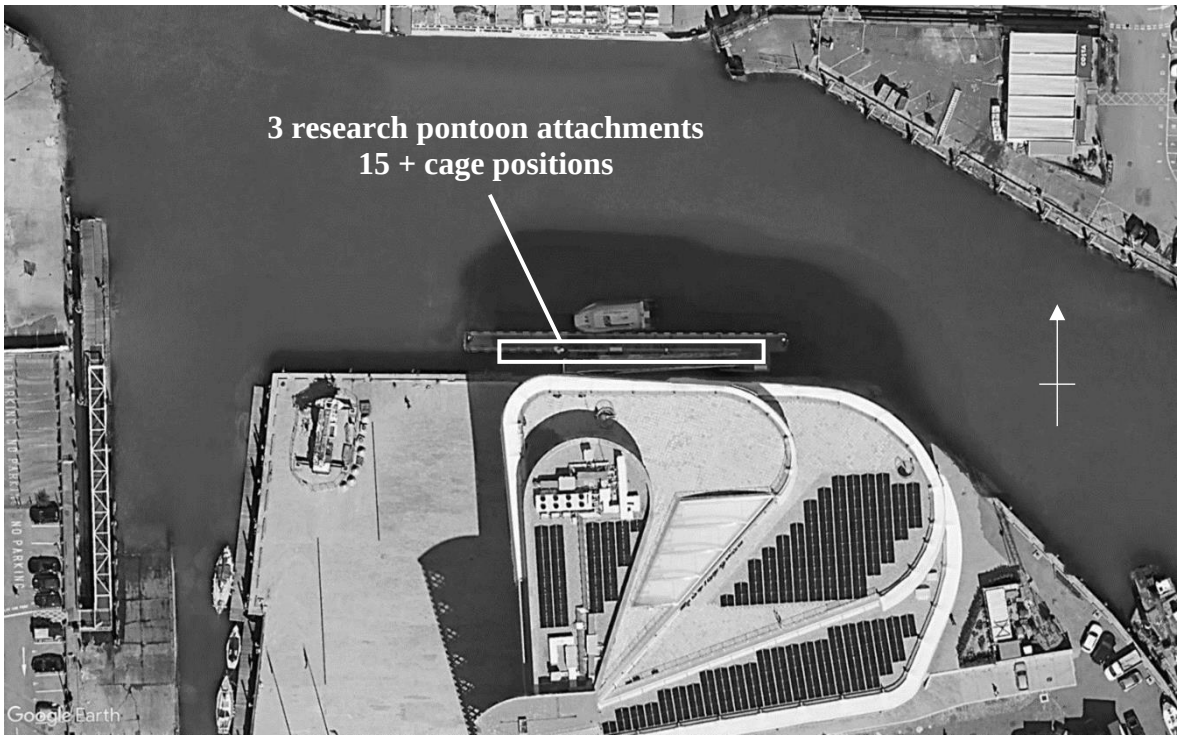
(A) Saxon Wharf



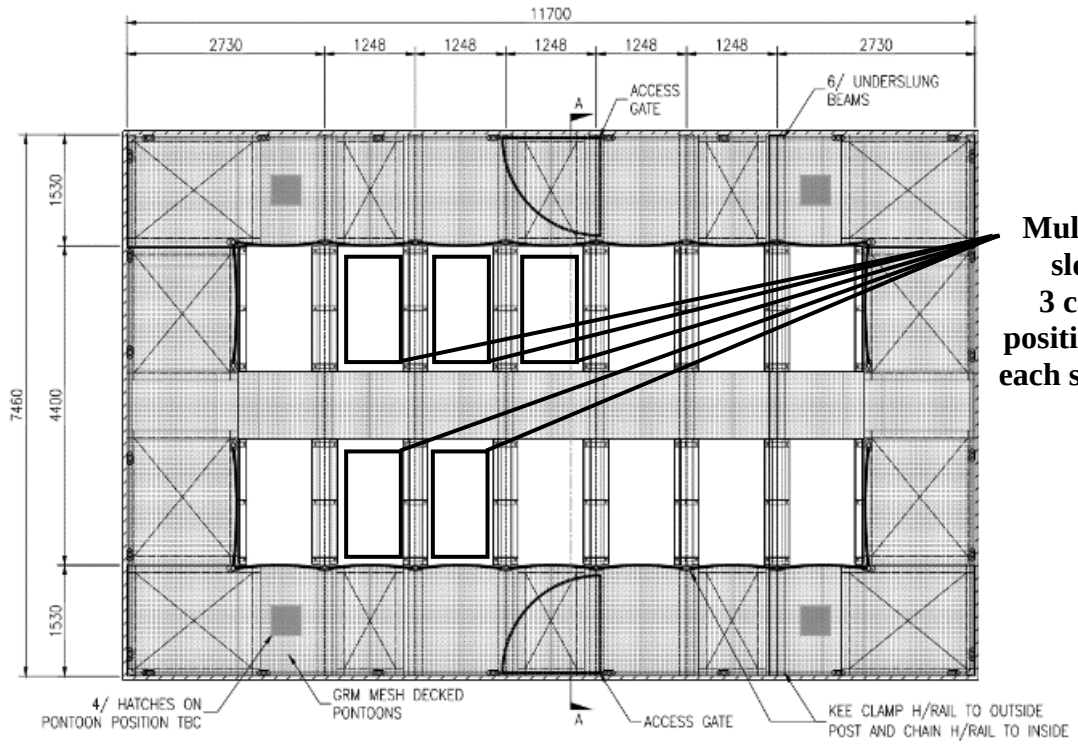
(B) Port Hamble Marina



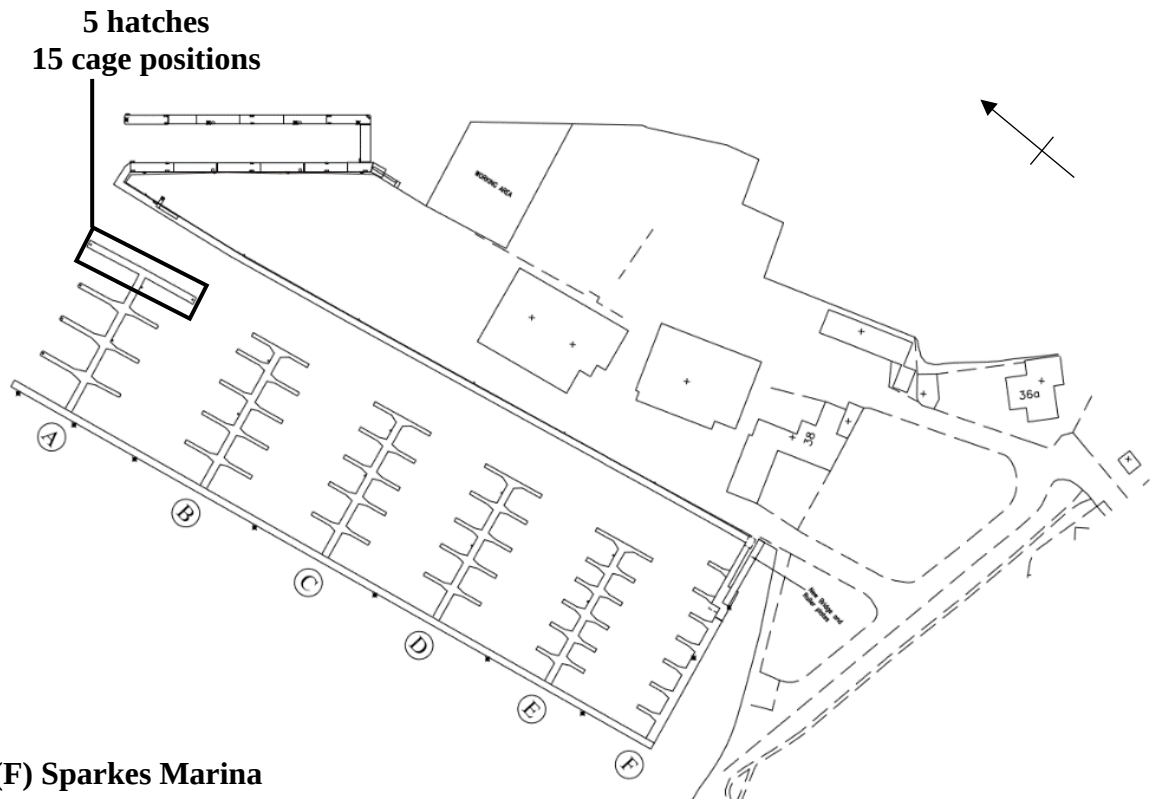
(C) Hamble Point Marina



(D) Camber Dock, Portsmouth Harbour



(E) University of Portsmouth research platform



(F) Sparkes Marina

Figure 3.4. Broodstock cage locations, shown with solid boxes, within (A) Saxon Wharf, River Itchen, (B) Port Hamble Marina, River Hamble, (C) Hamble Point Marina, River Hamble, (D) Camber Dock, Portsmouth Harbour, (E) University of Portsmouth Research Platform, Langstone Harbour and (F) Sparkes Marina, Chichester Harbour. Sources: Marina Developments Limited (Oliver Witt) and Google Earth. See Figure 3.5 for reference to location in relation to the wider Solent.

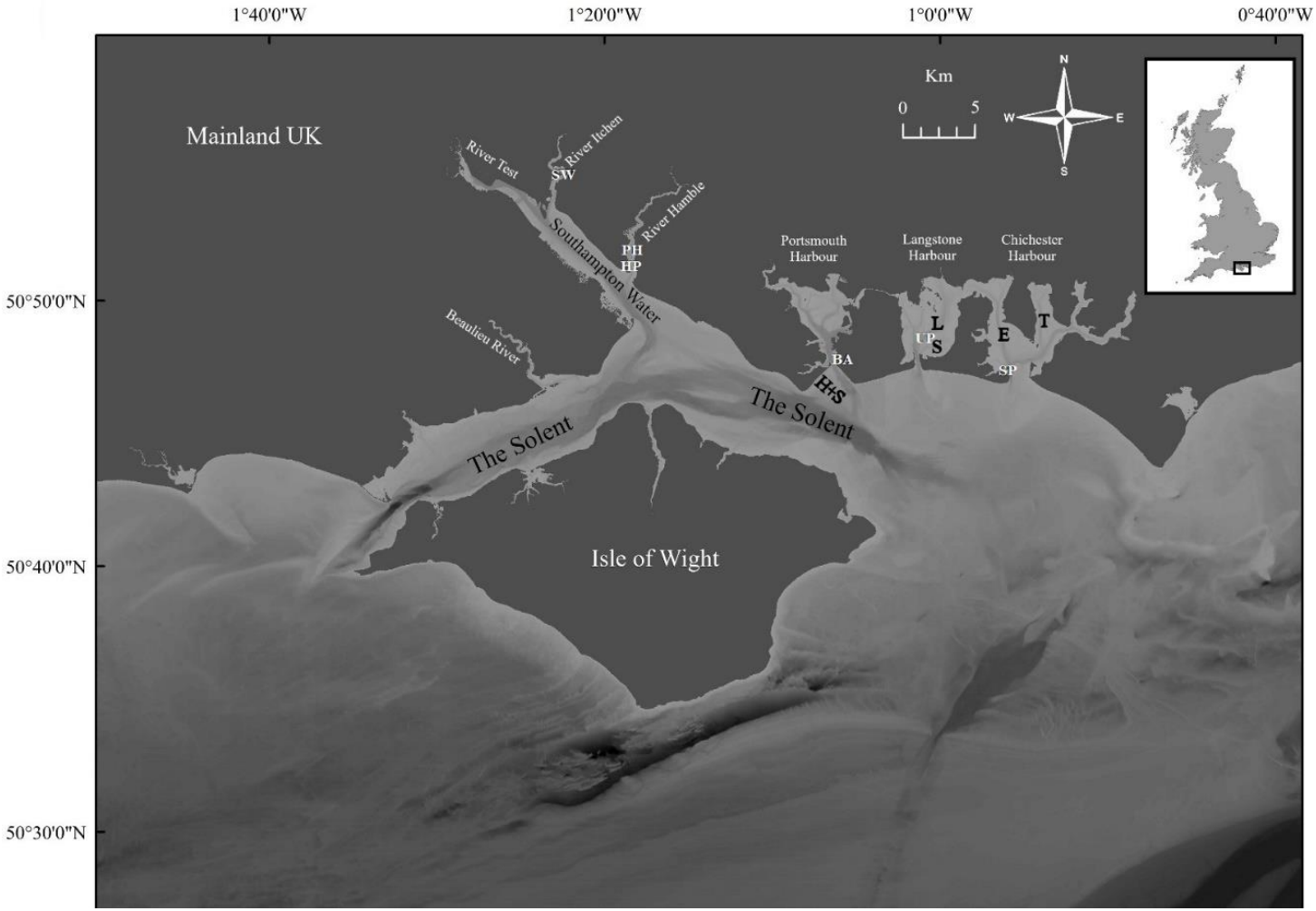


Figure 3.5. Map of the wider Solent denoting original fishery locations (Hamilton and Spitsand - H+S, Langstone Channel - L, Sinah Lake - S, Emsworth and Thorney Channels - E, T - black text) and broodstock cage marina locations (Saxon Wharf - SW, Port Hamble - PH, Hamble Point - HP, Camber Dock in Portsmouth Harbour - BA, University of Portsmouth Research Platform in Langstone Harbour - UP and Sparkes marina - SP) in white text.

3.2.3. Broodstock mortality

3.2.3.1. Year 1: May 2017 - April 2018

Cages at all locations were monitored monthly from May to October 2017, followed by sampling in January and April 2018. At each sampling interval, all cages were removed and the majority of the fouling epibiont community was removed from the oysters and cages using a pressure-washer (Kärcher™ K4 Premium Eco) to easily distinguish between live and dead oysters. Each oyster within the micro-reef modules was monitored and recorded as live or deceased. Those with open valves and no flesh present within the internal cavity were immediately classified as deceased. Those that displayed gasping behaviour (valves slightly open/slight movement) had both valves squeezed and released three times, if this elicited any closing response the individual was classified as living, and those that remained unresponsive and / or open were classified as deceased. Those that remained closed as the cages were removed from the water were checked by attempting to open the two valves by gently pushing/sliding them in opposite directions to assess whether there was a live oyster, or a mud filled cavity. Any oysters that remained closed and were not filled with mud were classified as living.

All deceased individuals were replaced using live oysters from cages designated for stock replenishment at the respective locations. Each location began the trial with five full-density, five half-density and two to four replenishing cages, dependant on stocks supplied at time of delivery to each location. The initial stock replenishing cages were used at all sites from May to July 2017, after this period two of the five full-density cages were removed from the monitoring population and were used to replenish the remaining three full-density cages. This was repeated for the half-density populations and three cages were replenished for both densities until October 2017. Due to mortality and logistical ease, the monitoring of full-density populations concluded in October 2017, the oysters were then distributed

amongst the remaining cages to replenish the three monitored half-density cages, for which the monitoring continued until April 2018. Due to logistical or weather-related issues with boat access, the population situated in Langstone Harbour were not monitored during August 2017 or January 2018. All other locations were monitored at all time points throughout the trial.

3.2.3.2. Year 2: May 2018 - October 2018

The sampling in year two, took place between May 2018 and October 2018, six cages were restocked as half-density populations, at each location. The impact of pressure-washing on oyster survival was monitored by separating the populations at each location into two sets of three cages, one set of which were pressure-washed with the oysters and micro-reef structures *in situ* to ensure exposure to high pressure and / or freshwater. In the second group the micro-reef structures, containing oysters, were removed prior to the pressure-washing of the cages themselves. This was conducted to remove epibionts and maintain the same flow rate into these cages as with those washed with the oysters *in situ*. Pressure-washing was conducted monthly and survival was recorded at the conclusion of the six-month sampling period (October 2018). It became apparent that a mass mortality event had occurred within all cages at Saxon Wharf between the monitoring time points of May and June 2018. Therefore, any results obtained for the impact of pressure-washing would be confounded by this external factor and as a result, the treatment was not continued after May 2018. Due to a lack of access to freshwater, cages and oysters situated on the University of Portsmouth's research platform were washed using pressurised seawater via the deck hose on board RV Chinook II for all months other than August, when cages were transferred to the Institute of Marine Sciences (IMS) and pressure-washed using freshwater, as mentioned previously.

3.2.4. Condition Index Year 2: October 2018

Oysters sampled for analysis of condition index (CI) were collected during the final monitoring of the pressure-washed and unwashed half-density broodstock cages at the six sample locations in October 2018. Due to a mass mortality event at Saxon Wharf at the beginning of the trial only unwashed oysters were sampled for CI from this location.

Condition index was performed according to the methodology in Culloty *et al.* (2004, p 45), whereby the oysters were shucked, and the internal tissue was separated from the two valves, taking care to ensure that all of the adductor muscle tissue was removed from both valves. The tissue was then rinsed in a beaker of distilled water, blotted dry and wrapped in aluminium foil. The two valves were placed in a large glass petri dish lid, or base, with the parcel of tissue placed in a small glass petri dish lid, or base, all samples were then placed in a drying oven at 60 °C for 48 h to obtain dry tissue weight. The calculation used by Walne & Mann (1975) and Lucas & Beninger (1985) (cited in Helmer *et al.* (2019)) was then used to determine CI:

$$\textit{Condition index} = \textit{Dry tissue weight} / \textit{Dry shell weight} \times 100$$

3.2.5. Size at death

With the hypothesis that firstly, when larger individuals are placed into the micro-reef structures, they would be unable to open and feed efficiently, or that they would be reaching the end of their natural life cycle. Secondly, if mortality was occurring evenly across all size classes then the cause may be an environmental stressor or related to prevalence of disease. Oysters that did not survive the trial (3.2.3.2) were collected in November 2018 and returned to the laboratory to determine whether the mortality experienced within the cage systems was size / age dependant, or a product of the micro-reef structures.

Measurements were collected to the nearest 1 mm for the maximum shell length, width and depth according to Helmer *et al*. (2019) (as in 2.2.3). Whole wet weight was not collected due to the absence of internal flesh in these samples. These data were then compared with the morphometric analysis of the original fishery population from 2016, in 2.2.3, to determine if the deceased individuals were significantly larger than the initial population. Frequency data for maximum shell length and width were arrayed into 5 mm groupings and maximum shell depth was arrayed into 1 mm groupings.

3.2.6. Environmental parameters

3.2.6.1. Temperature

Temperature (°C) within the cages was recorded by attaching a HOBO Pendant® Temperature/Light 64K Data Logger - UA-002-64 (Onset Computer Corporation, USA) to the mesh of the cage so that the logger was contained within internal area where the oysters were housed. Measurements were recorded every 15 minutes.

3.2.6.2. Nutrient analysis

Water samples were collected prior to the monitoring of the broodstock cage populations each month from May 2017 to October 2018 at the six marinas detailed in 3.2.2. All seawater was collected from the surface of the water column in close proximity to the broodstock cages and any seawater used to rinse any of the equipment, prior to sample preservation, was retained in a container on the pontoon as to not release this into the water column to be re-sampled. Firstly, a clean 20 ml luer-slip syringe (Thermo Scientific™) was used to pass 5 ml of sea water through a 0.2 µm sterile polyethersulfone (PES) syringe filter (Fisherbrand™) to remove any debris that may have been present within the filter mechanism, this process was repeated. The syringe was filled with 15 ml of seawater and 5 of the 15 ml was filtered into three separate 30 ml polypropylene universal containers (Fisher Scientific) to rinse any debris that was contained within them. This process was repeated for the second and third universal containers. The samples were then fixed by adding 200 µl zinc chloride ($ZnCl_2$) to the filtered seawater immediately on site. A precipitation that formed within the samples was dissolved with 1 M Hydrochloric acid (HCl). An AA500 and AS2 Auto Analyser (SEAL Analytical Ltd) was then used to quantify nitrite, nitrate, silica and phosphate concentrations. Sample analysis was delayed due to a series of unfortunate events that occurred, therefore, the results of this section of the study were not available for presentation in this thesis.

3.2.6.3. Further environmental records

Additional temperature, nitrite, nitrate, ammoniacal nitrogen, orthophosphate, silicate, turbidity, salinity, chlorophyll and dissolved oxygen data were obtained from the Environment Agency's (EA) Water Quality Data Archive (Environment Agency, 2019).

Data was available and selected that aligned with the 2017 sampling period from May 2017 - February 2018 and for the 2018 monitoring period of May 2018 - September 2018. Data was selected from samples that were collected at 0.2 m depth. Data was not available for March and April 2018 that aligned with the first years sampling (2017 into 2018 season), or for October and November 2018 that aligned with the end of the 2018 season. Nutrient data that was recorded on the EA database as "< n" was converted to a 0.0 µl result for the purpose of analysis as the recordings were negligible.

The EA sampling stations closest to the respective broodstock cage population was selected, as follows:

Saxon Wharf - R Itchen Kemps Boatyard (50° 54.8218'N 1° 22.667)

Port Hamble - Hamble Estuary Ferry Slipway (50° 51.4777'N 1° 18.7208)

Hamble Point - Hamble Estuary Ferry Slipway (50° 51.4777'N 1° 18.7208)

Portsmouth Harbour - Portsmouth Harbour Mouth (50° 47.5003'N 1° 6.6233)

Langstone Harbour - Nw Sinah Buoy, Langstone (50° 48.3752'N 1° 1.3272)

Sparkes Marina - Point 16 Fishery Buoy (50° 47.3461'N 0° 55.9604)

3.2.7. Statistical analysis

Statistical analysis was carried out in R (R Core Team, 2017), IBM® SPSS 25® Statistics 25 (IBM Analytics, USA) or PRIMER-e v. 6 (Clarke and Gorley, 2006). A General Linerar Model (GLM) with binomial error distribution was used to analyse the broodstock mortality in 2017 with marina, density and cage position as fixed effects and month as a factor.

Owing to non-normal distributions, a Kruskal-Wallis H test was used to analyse comparisons of mortality within the two cleaning treatments, condition index and size at death data, in 2018. This was also conducted to assess differences with the environemantal parameters in the central and eastern areas of the Solent in the first year of the trial.

The influence of environmental conditions on mortality in 2017 and 2018 as well as condition index in 2018 was assessed using the Principal Coordinated Analysis (PCO) available in PRIMER-e. Mortality data from 2017 were grouped into low (0 - 20 %), medium (20 - 40 %) and high (40 % +) mortality with site (central or eastern), marina (SW, PH, HP, BA, UP or SP) and density (high or low) as factors. Mortality data from 2018 were grouped into low (0 - 50 %), high (50 - 80 %) and very high (80 % +) mortality with site (central or eastern), marina (SW, PH, HP, BA, UP or SP) and treatment (pressure-washed or unwashed) as factors. Condition index data from 2018 were grouped into cohorts of 0 - 2, 2 - 4, 4 - 6 and 6 + with site (central or eastern), marina (SW, PH, HP, BA, UP or SP) and treatment (pressure-washed or unwashed) as factors.

3.3. Results

3.3.1. Broodstock mortality

3.3.1.1. Year 1: May 2017 - April 2018

Initially, during May and June 2017, average monthly mortality for broodstock at all location was extremely low (both months 5.6 % mean for all locations), with the lowest mortality of 2.7 % recorded in Saxon Wharf (SW) during May, and the highest mortality observed in Langstone Harbour (UP) in June, 8.8 % (Fig. 3.6).

Mortality peaked in July at an average of 21.4 % across all populations, approximately four times greater than that of the previous two months. Port Hamble (PH) experienced the greatest mortality with an average of 36.9 %. Hamble Point (HP), the closest population geographically, experienced fewer deceased oysters with average of 20.8 %.

A decrease in average mortality was observed the following month of August with an average for all locations of 17.8 %, with the population at SP experiencing greater mortality than all other locations, 31.3 %. The mortality in September continued to decline to 16.4 % on average, with the UP population experiencing a greater mortality than all other locations, 32.6 %, reflecting the lack of sampling and restocking the previous month.

The mortality reached its lowest point in October at an average of 13.6 %, during this period the BA population experienced the most mortality at 20.8 %. After the winter of 2017 populations of half-density cages, were once again monitored in January 2018 where the average mortality per cage for all locations had risen to 30.2 %. The population at BA experienced greater mortality than all other locations, 55 %. A slight decrease, to 27.2 %, was observed across all locations moving into April 2018. Again, due to the lack of monitoring and restocking the month prior to this, the UP population experienced the greatest mortality, 45.6 %.

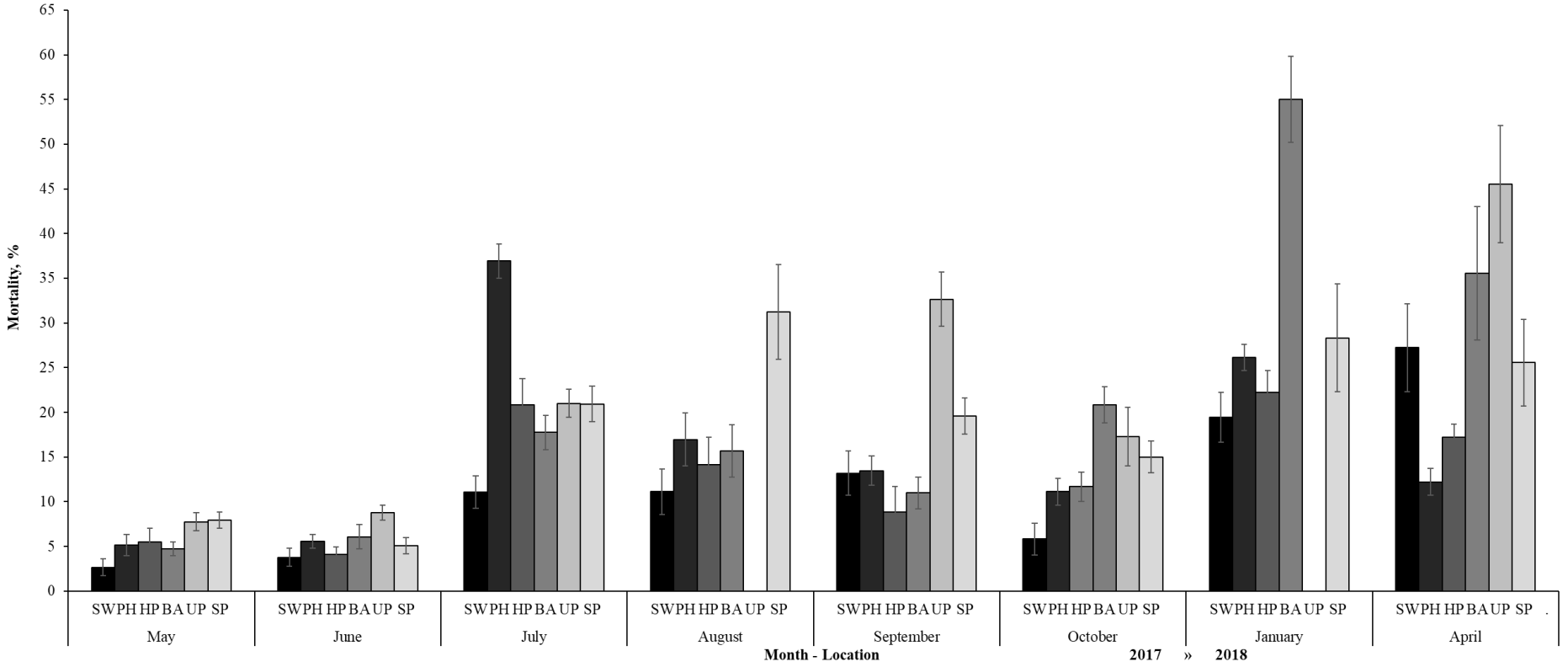


Figure 3.6. Percentage mortality (mean ±SE) for all broodstock cages at the six locations throughout the spawning season from May - October 2017 and after the 2017/18 winter period. Ten broodstock cages containing 900 oysters were monitored at each location in May, June and July 2017, six broodstock cages containing 540 oysters were monitored in August, September and October 2017, and three cages containing 180 oysters were monitored in January and April 2018. Locations not monitored for particular months are shown by X. Lower-case data labels indicate significant differences between locations during the same month ($p < 0.05$). Codes: SW - Saxon Wharf, PH - Port Hamble, HP - Hamble Point, BA - Portsmouth Harbour, UP - Langstone Harbour, SP - Sparkes Marina.

The percentage mortality within full-density cages, pooled from all locations, did not differ significantly from that within the half-density cages ($p > 0.05$). Mortality within both densities varied by < 2.5 % throughout the year and the greatest difference was observed in September, 2.4 % (Fig. 3.7).

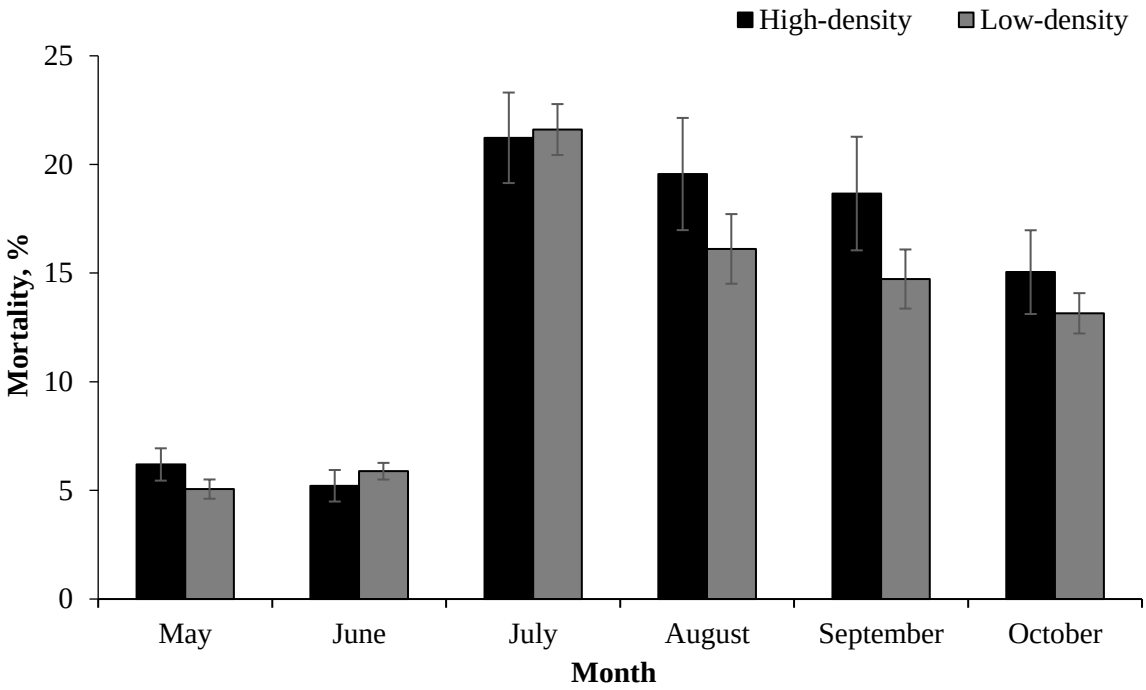
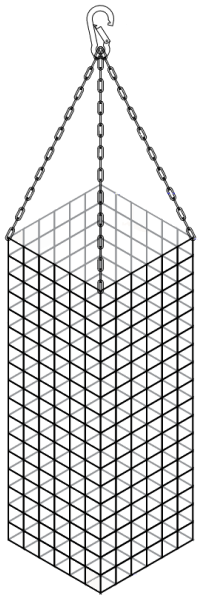


Figure 3.7. Percentage mortality (mean ±SE) of oysters in full- and half-density cages pooled from all six sampling locations at each month during the six-month period between May and October 2017 where a comparison is available, after this period only half-density cages were trialled.

Position within the cages was determined to be a significant factor in influencing mortality ($p < 0.001$) and visual representations of the mortality within full and half-densitiy cages are shown in Figures 8 and 9 for the total period and on a monthly basis in Figures 10 and 11.



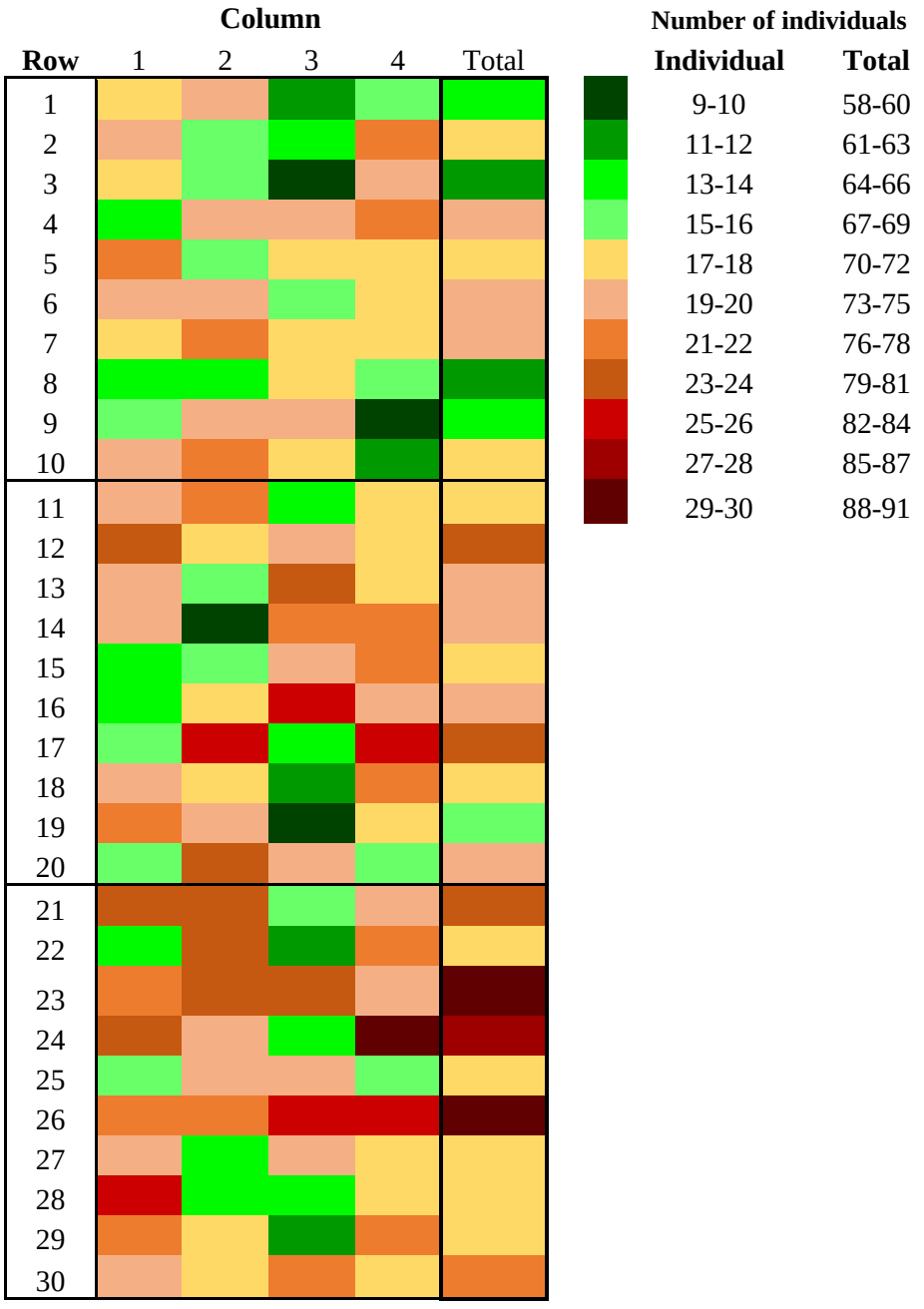


Figure 3.8. Mortality within micro-reef structures for each individual position, within each column, for full-density broodstock cages at all locations, across all months of the spawning season (May - October 2017). Total mortality represents that of each row across all four columns (n / 564). Each row within the micro-reef unit was occupied by an oyster.

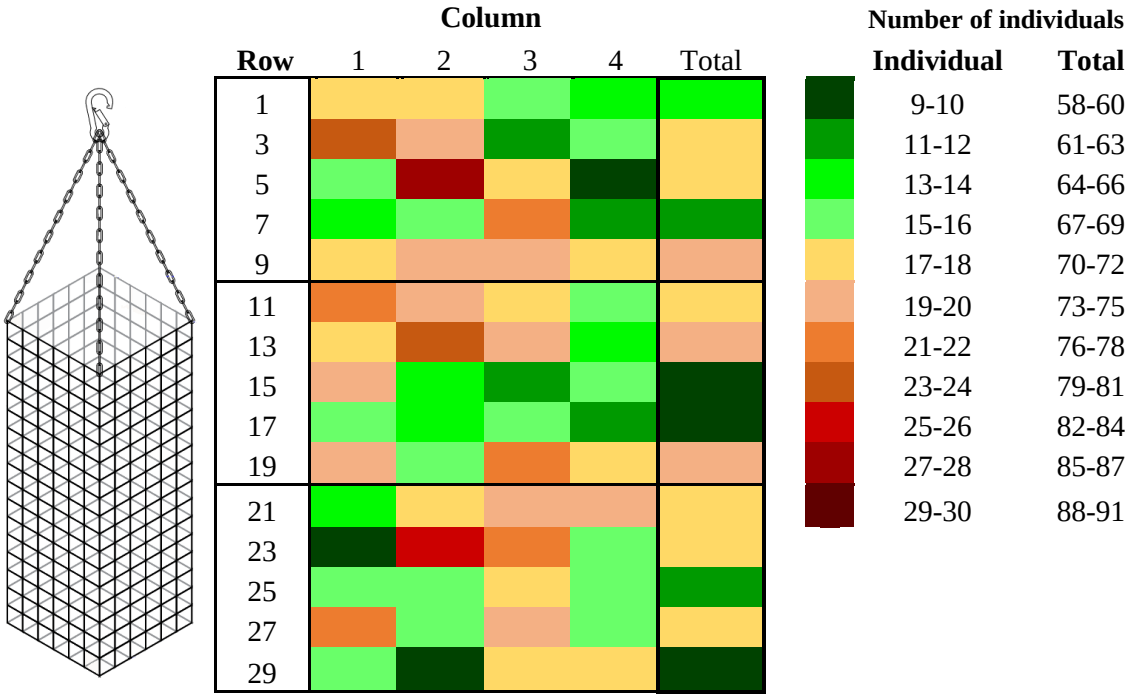


Figure 3.9. Mortality within micro-reef structures for each individual position, within each column, for half-density broodstock cages at all locations, across all months of the spawning season (May - October 2017). Total mortality represents that of each row across all four columns (n / 564). Every other row within the micro-reef unit was occupied by an oyster.

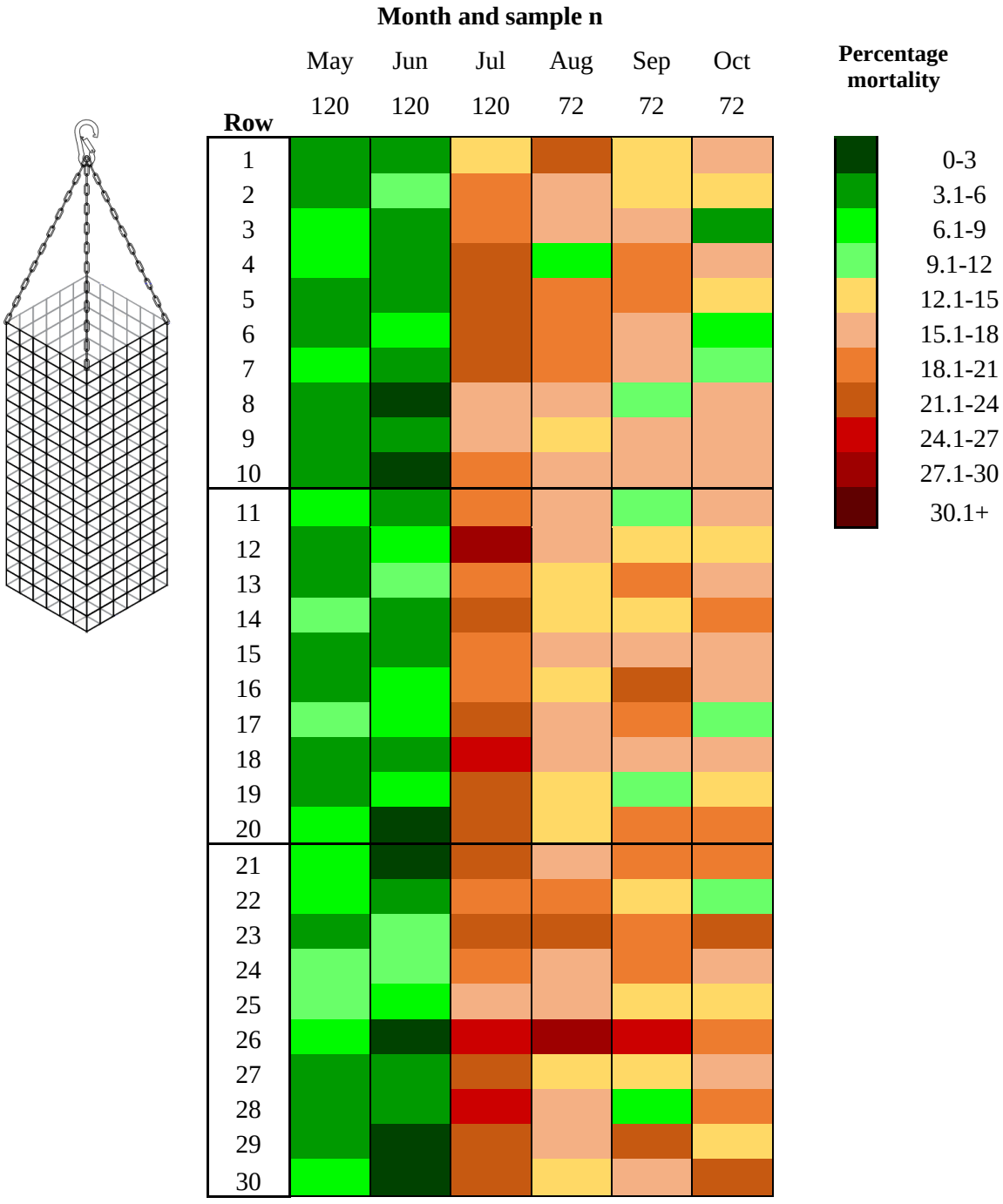


Figure 3.10. Percentage mortality within micro-reef structures for each row within all columns, for full-density cages at all locations, across each month of the spawning season (May - October 2017). Sample size for each individual position for the respective month.

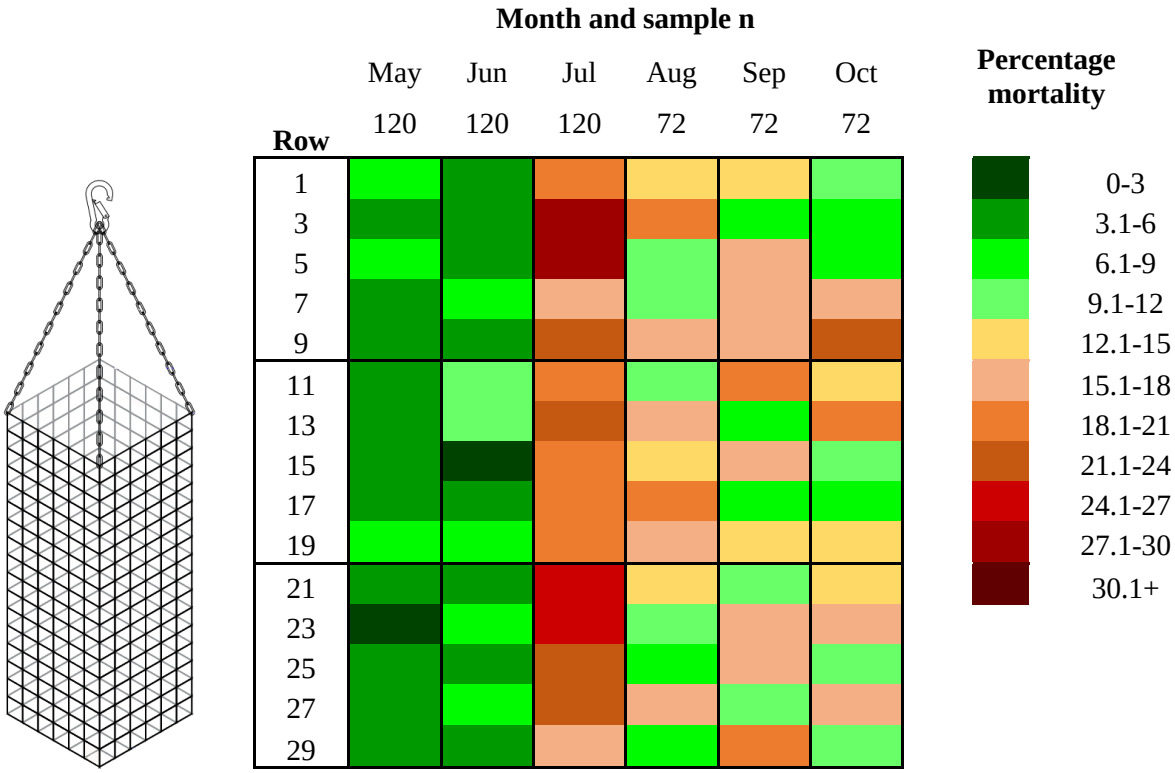


Figure 3.11. Percentage mortality within micro-reef structures for each row within all columns, for half-density cages at all locations, across each month of the spawning season (May - October 2017). Sample size for each individual position for the respective month.

3.3.1.1. Year 2: May - October 2018

A mass mortality event occurred at a variety of locations along the River Itchen between May and June at the beginning of the 2018 trial, including areas used for the wider project but not monitored as part of this study, for this reason no oysters or cages were pressure-washed at the SW location. The mortality for the unwashed cages at this site was 95.3 ±1.8 % (mean ±SE), and was significantly higher than the unwashed cages at all other locations (Kruskal-Wallis H test, H (10) = 25.314, p = 0.005). No significant differences were observed in the mortality experienced within the unwashed cages between the other locations ($p < 0.05$).

Mortality within pressure-washed populations was greatest at SP, 87.2 ±2.4 %, and was significantly higher than BA, 64.2 ±2.5, and UP, 62.2 ±5.6 %, populations ($p < 0.05$), however, this was not significantly greater than the PH or HP populations, 80 ±4.4 and 78.3 ±1.7 %, respectively ($p > 0.05$). There was no significant difference between any of the PH, HP, BA and UP pressure-washed populations ($p > 0.05$).

The mortality within pressure-washed populations was greater than that of unwashed populations at PH, HP, UP and SP ($p > 0.05$), however, the only significant difference was observed within the SP population ($p < 0.05$). The mortality within the pressure-washed population at BA was lower than that of the unwashed population, however, the difference was not significant ($p > 0.05$) (Fig. 3.12).

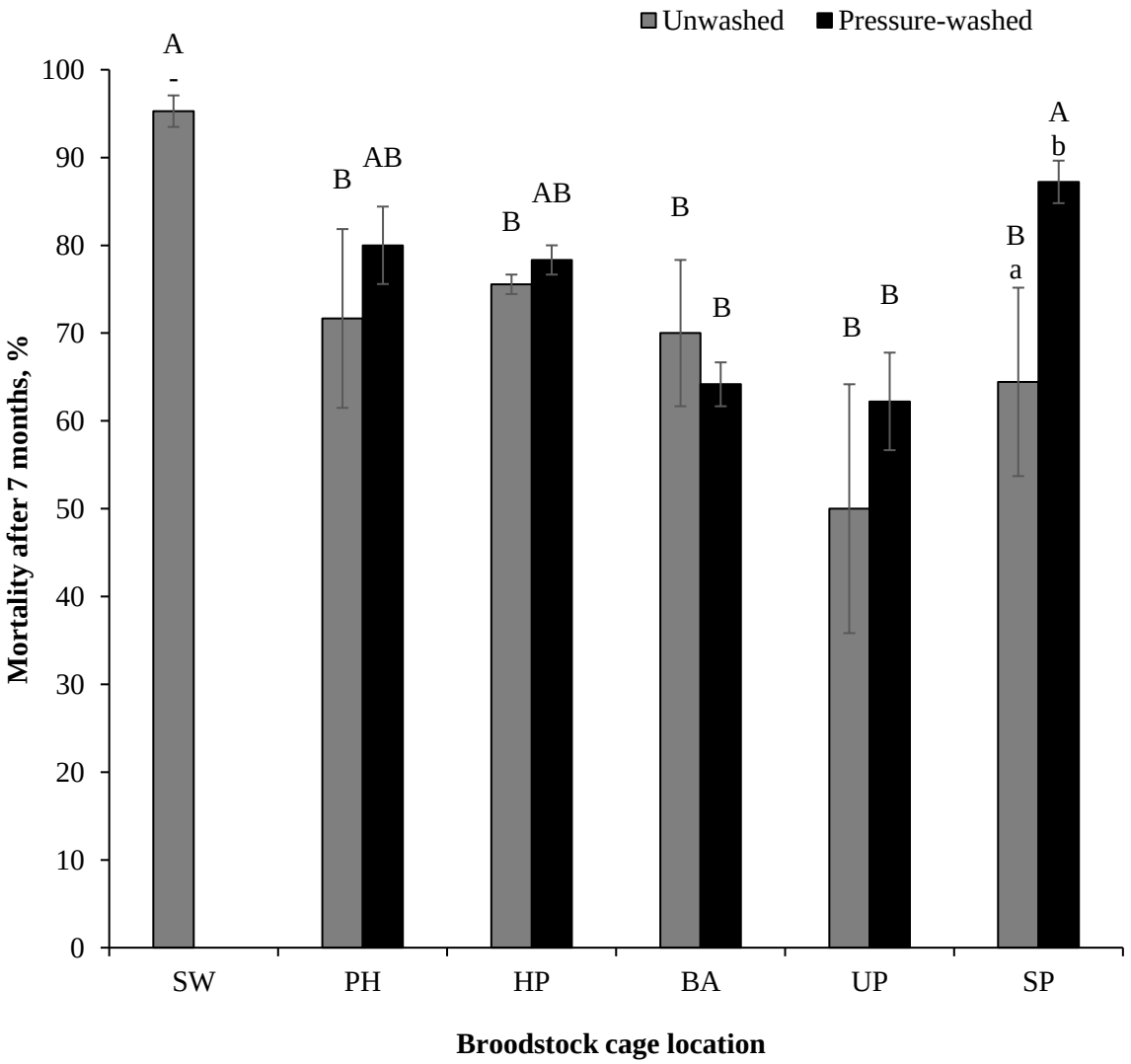


Figure 3.12. Percentage mortality (mean ±SE) of oysters in unwashed and pressure-washed cages at the six sampling locations at the conclusion of the seven-month period that began in May and ended in November 2018. A mass mortality event occurred at Saxon Wharf (SW), so all cages were left unwashed (n = 360). Pressure-washed and unwashed populations were tested at all other locations (n = 180 / treatment). Upper-case data labels indicate any significant difference between the same treatments at different locations and lower-case data labels indicate any significant difference between the treatments at the same locations ($p < 0.05$).

3.3.2. Broodstock condition index

The remaining population at SW at the end of the 2018 trial were found to have the greatest condition index (CI) of all the sites, 5.6 ±0.7 (mean ±SE) and was 0.8 higher than that of the UP population, however this difference was not significant (Kruskal-Wallis, H (5) = 7.758, p = 0.526). The condition index (CI) of both populations were significantly greater than that of all other locations ($p < 0.05$). There was no significant difference between the PH and HP or the PH and SP populations ($p > 0.05$), however, the BA population did have a significantly higher CI than the PH population ($p < 0.05$) but this was not significantly higher than either the HP or SP populations ($p > 0.05$) (Fig. 3.13).

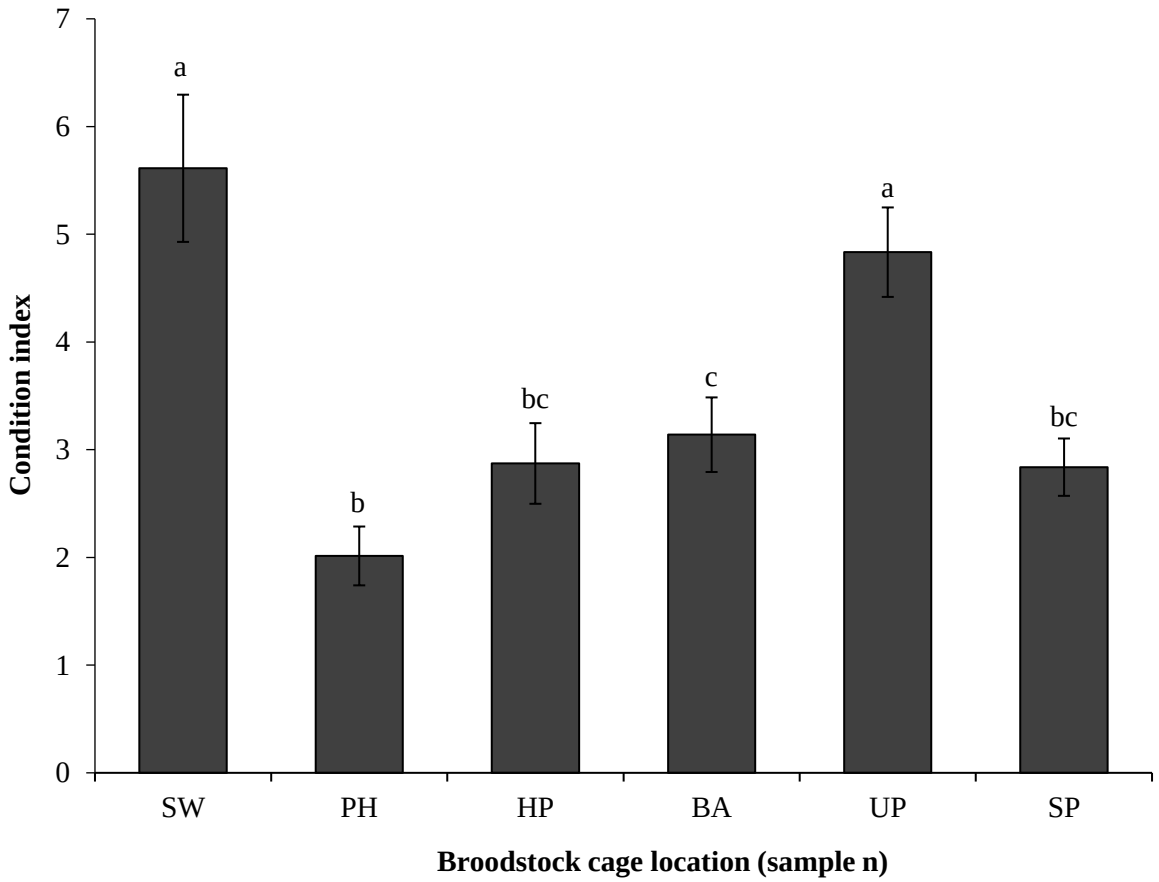


Figure 3.13. Condition index (CI) (mean ±SE) results obtained during November 2018 from the mature oysters in the broodstock cages situated at six locations across the Solent. Lower-case data labels indicate significant differences between the CI of oysters at different locations ($p < 0.05$). Sample n = 10 for SW and n = 20 for all other locations.

The condition index of oysters exposed to pressure-washing at HP, BA, UP and SP was 1.0, 0.5, 1.0 and 0.1 greater than that of oysters not exposed to the pressure-washing treatment at their respective locations, however, none of the values were significantly different ($p > 0.05$) (Fig. 3.14). The population of oysters exposed to pressure washing at PH was 0.2 lower than the non-exposed to the pressure-washing treatment, again these values were not significantly different from one another ($p > 0.05$). No comparison was available for the SW populations. No significant difference was observed between the two treatments when all populations at all locations were pooled together.

The greatest CI, 5.6 ±0.7, was observed within the population of unwashed oysters at SW, an area that experience a mass mortality event early in 2018, thus no pressure-washed oysters were present. The comparison of unwashed populations at different locations revealed a significant difference with the increased CI values at SW and UP (4.3 ±0.7) populations, compared with the PH, HP, BA and SP populations ($p = < 0.05$).

The CI of the UP pressure-washed population was the highest for this treatment (5.3 ±0.5) and in comparison, the PH population this increase was significant ($H = 2.657$, $p = 0.008$). Despite the CI of the UP population being 1.9 higher than that of the HP and BA populations (both 3.4 ±0.6), and 2.4 higher than the SP population (2.9 ±0.4), there was no significant difference between these locations ($p > 0.05$). There was also no significant difference between the CI of the pressure-washed oysters at PH, HP, BA or SP ($p > 0.05$).

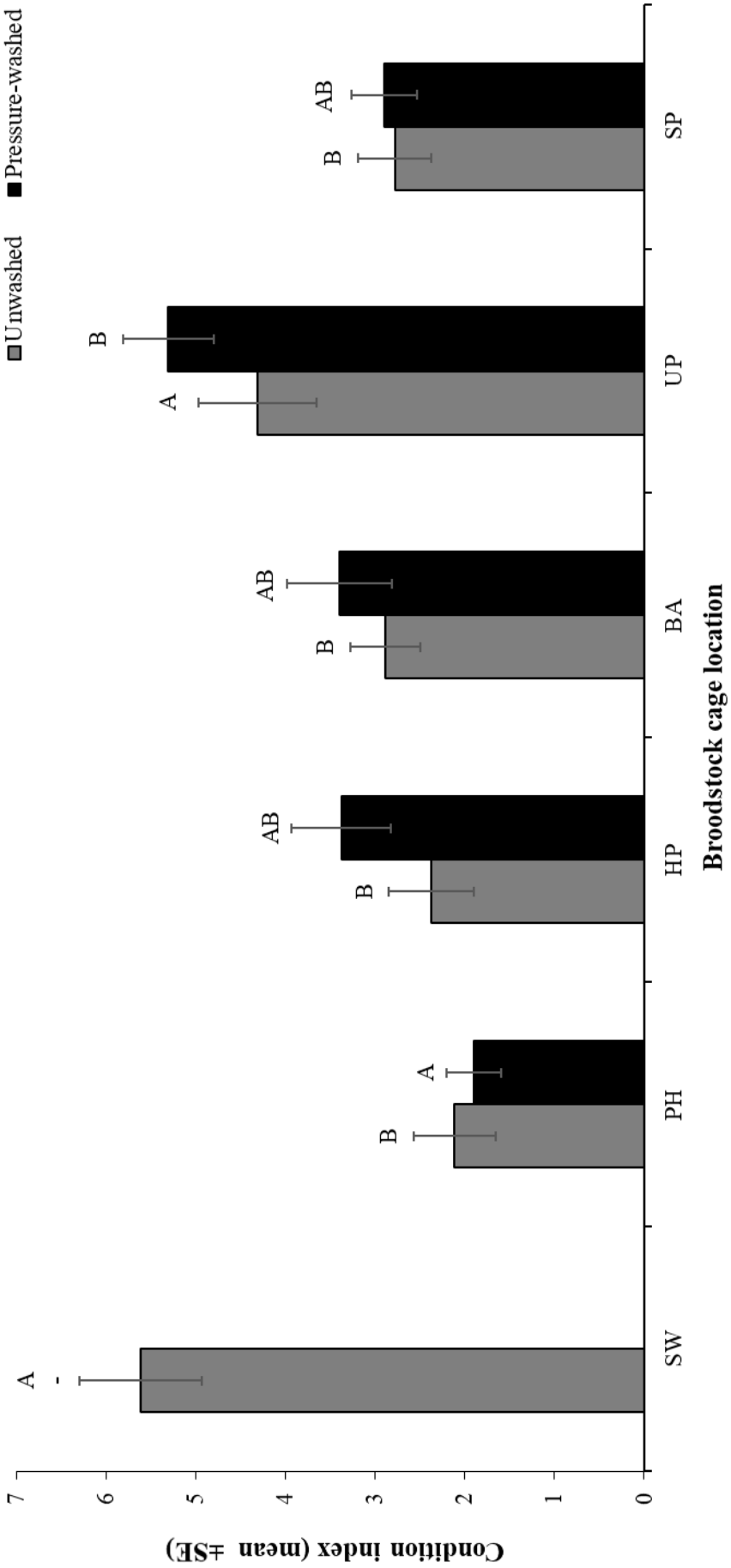


Figure 3.14. Condition index (CI) (mean ±SE) results obtained during November 2018 from the mature oysters in the broodstock cages situated at six locations across the Solent. A mass mortality event occurred at Saxon Wharf (SW) so only unwashed oysters were able to be sampled. Ten oysters from pressure-washed and unwashed populations were tested at all other locations. Upper-case data labels indicate significant differences between the same treatment at different locations. The absence of lower-case data labels indicates that there was significant difference between different treatments at the same locations.

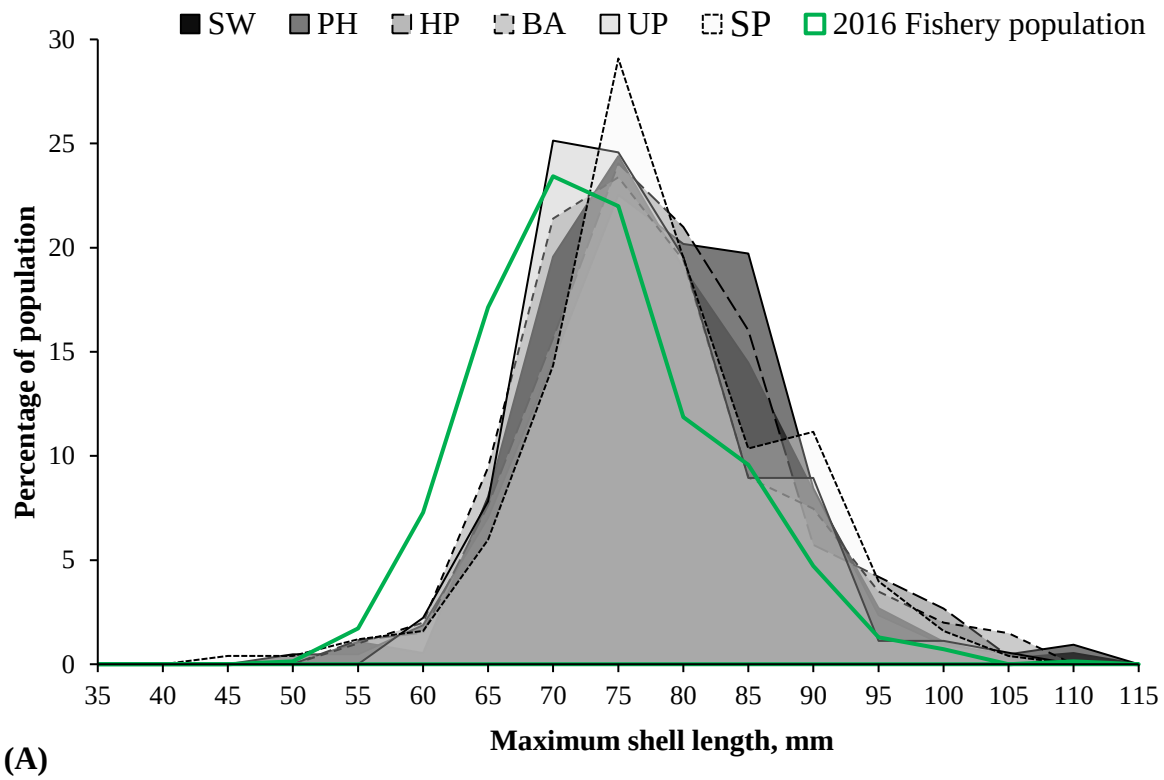
3.3.3. Size at death

The most frequent maximum shell length (MSL) for the original 2016 fishery live population was 70 - 74.9 mm (23.4 %) (Fig. 3.15A - B). The most frequent MSL for the 2018 deceased broodstock populations at Saxon Wharf (SW), Port Hamble (PH), Hamble Point (HP), Portsmouth Harbour (BA), and Chichester Harbour (SP), individually (Fig. 3.23A), and the mean of all six locations combined (Fig. 3.15B) was 5 cm greater at 75 - 79.9 mm (24.4, 22.5, 24.0, 23.4, 29.1 and 24.5 %, respectively). The most frequent MSL for the 2018 population in the Langstone Harbour (UP) population was the same as the 2016 fishery population at 70 - 74.9 mm (25.1 %) (Fig. 3.15A). Oysters > 75 mm in length accounted for 50.3 % of the 2016 fishery population, the same size range of deceased broodstock oysters accounted for an average of 71.2 % across all six locations. The mean of deceased oysters accounted for a greater percentage than those within the 2016 fishery population for all size groupings > 75 mm. The average MSL of the 2016 fishery population, 71.5 ±0.3 mm, was significantly lower than all 2018 broodstock locations (Kruskal-Wallis H test, H (6) = 130.222, $p < 0.001$) and the pooled broodstock average, 75.9 ±0.2 (H = 120.225, $p < 0.001$). The average MSL of the SW population did not significantly differ from any of the other broodstock locations ($p > 0.05$), the PH, HP and SP population average MSLs were significantly greater than that of the UP population ($p < 0.05$), however, the BA population was not ($p > 0.05$). The PH and HP populations average MSL was also significantly greater than that of the BA population ($p < 0.05$), however, the SP population was not ($p > 0.05$).

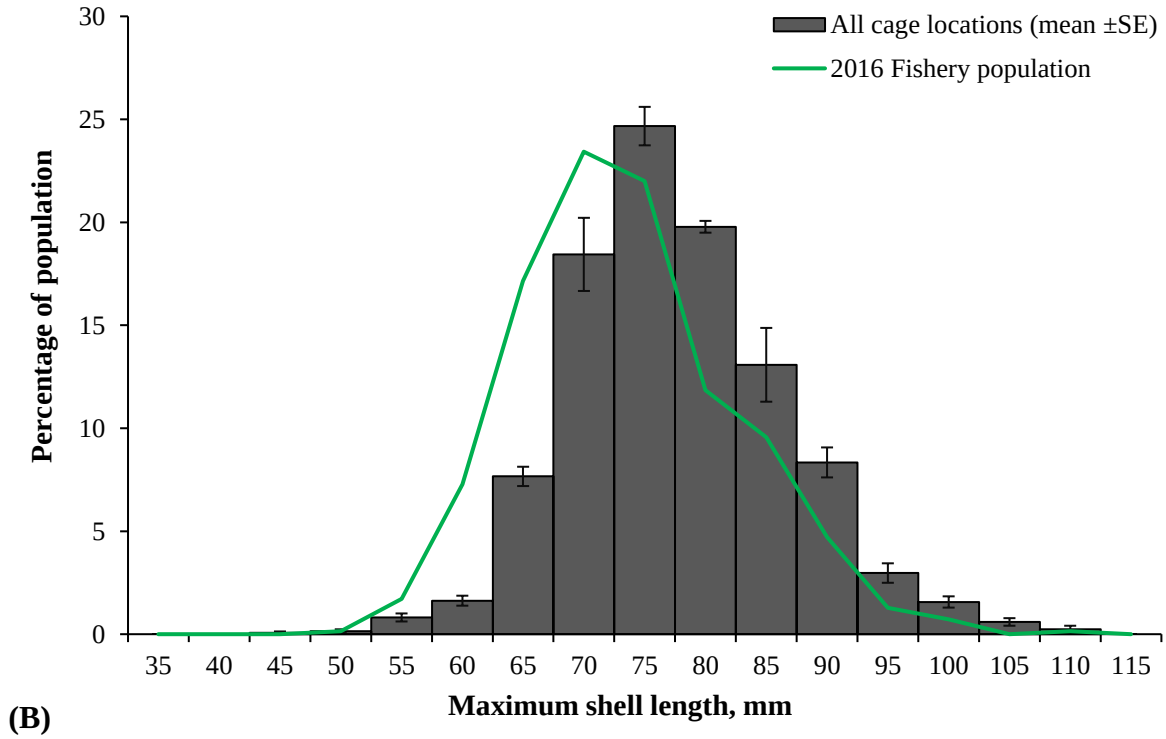
The most frequent maximum shell width (MSW) for the original 2016 fishery live population was 70 - 74.9 mm (21.6 %) (Fig. 3.16A - B). The most frequent MSW for the 2018 deceased broodstock populations at SW, PH, HP and SP (Fig. 3.16A) was 5 cm greater at 75 - 79.9 mm (28.4, 23.9, 23.3 and 25.1 %, respectively). The most frequent MSW for the

deceased broodstock populations at BA and UP (Fig. 3.16A), and the mean of all six locations combined (Fig. 3.16B) was the same as the 2016 fishery live population at 70 - 74.9 mm (24.9, 29.1 and 23.7 %, respectively). Oysters > 75 mm in width accounted for 59.3 % of the 2016 fishery populations, the same size range of deceased broodstock oysters accounted for an average of 56.1 % across all six locations. No significant difference was observed between the average MSW of any of the populations (H (6) = 11.012, p = 0.088), however the average MSW of the 2016 fishery population was significantly greater than that of the pooled population from the 2018 broodstock cages (H = 4.177, p = 0.041).

The most frequent maximum shell depth (MSD) for the original 2016 fishery live population was 20 - 20.9 mm (11.1 %) (Fig. 3.17A - B). The most frequent MSD for the 2018 deceased broodstock populations at SW, HP, BA, UP and SP (Fig. 3.17A), as well as the mean of all six locations combined (Fig. 3.17B), was 5 cm greater than that at 25 - 25.9 mm (15.0, 16.4, 8.5, 13.4, 12.0 and 12.8 %, respectively). The two most frequent MSDs for the deceased broodstock oysters at PH population were 3 and 5 cm greater than the 2016 fishery population at 23 - 23.9 and 25 - 25.9 mm (both 11.7 %) (Fig. 3.17A). The mean percentage of deceased individuals at 25 - 25.9 cm depth (12.8 %) was 4.2 % greater than the next most frequent size of 26 - 26.9 cm. Oysters > 20 mm in depth accounted for 65.4 % of the 2016 fishery populations, the same size range of oysters accounted for an average of 91.9 % of those deceased across all six locations. The average MSD of the 2016 fishery population, 21.3 ±0.2 mm, was significantly lower than all broodstock locations (Kruskal-Wallis H test, H (6) = 358.729, $p < 0.001$) and the pooled broodstock average, 25.7 ±0.1 (H = 343.392, $p < 0.001$). The average MSD of the SW, BA, UP and SP populations were significantly greater than that of the PH population ($p < 0.05$), however, the HP population was not ($p > 0.05$). The MSD of the SW and UP populations were also significantly greater than the HP population ($p < 0.05$), however, BA and SP populations were not ($p > 0.05$).

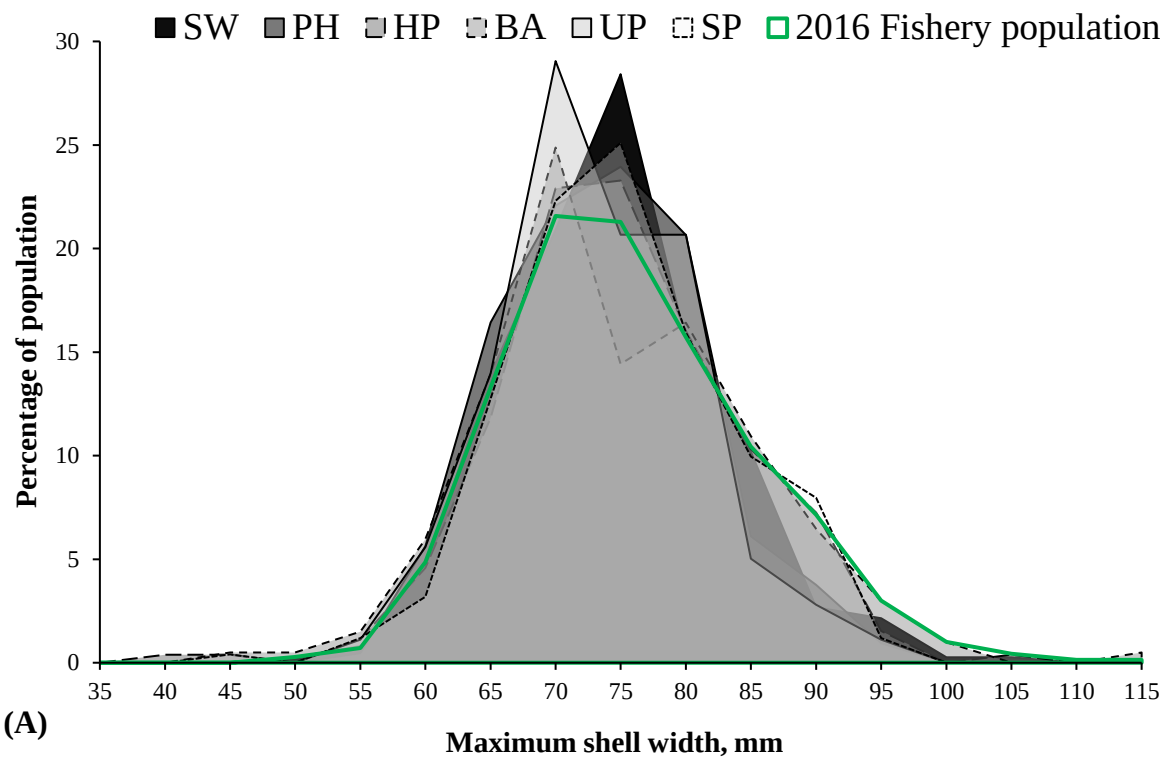


(A)



(B)

Figure 3.15. (A) Maximum shell length of deceased individuals collected from each broodstock cage location in October 2018 in comparison to the initial 2016 fishery population used to stock the cages. Data collated into 5 mm arrays. (B) Maximum shell length of deceased individuals from all broodstock cage locations collected in October 2018 (mean ±SE) in comparison to the initial 2016 fishery population used to stock the cages.



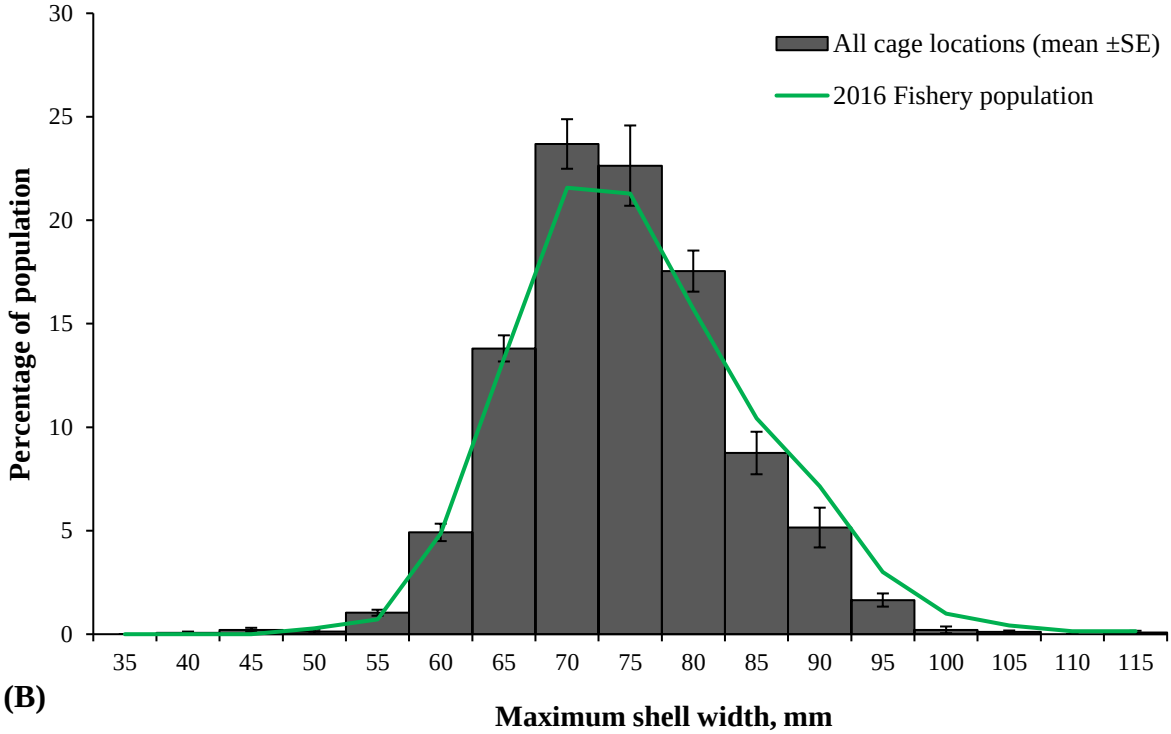
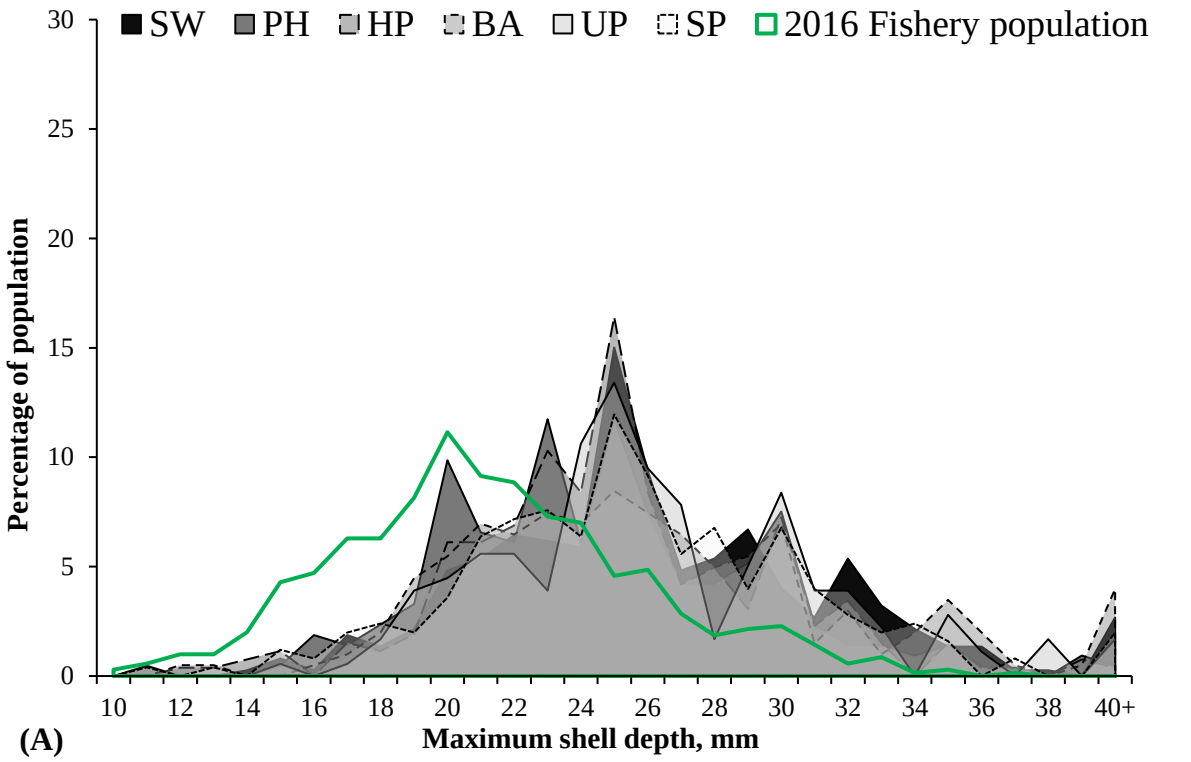


Figure 3.16. (A) Maximum shell width of deceased individuals collected from each broodstock cage location in October 2018 in comparison to the initial 2016 fishery population used to stock the cages. Data collated into 5 mm arrays. (B) Maximum shell width of deceased individuals from all broodstock cage locations collected in October 2018 (mean ±SE) in comparison to the initial 2016 fishery population used to stock the cages. Data collated into 5 mm arrays.



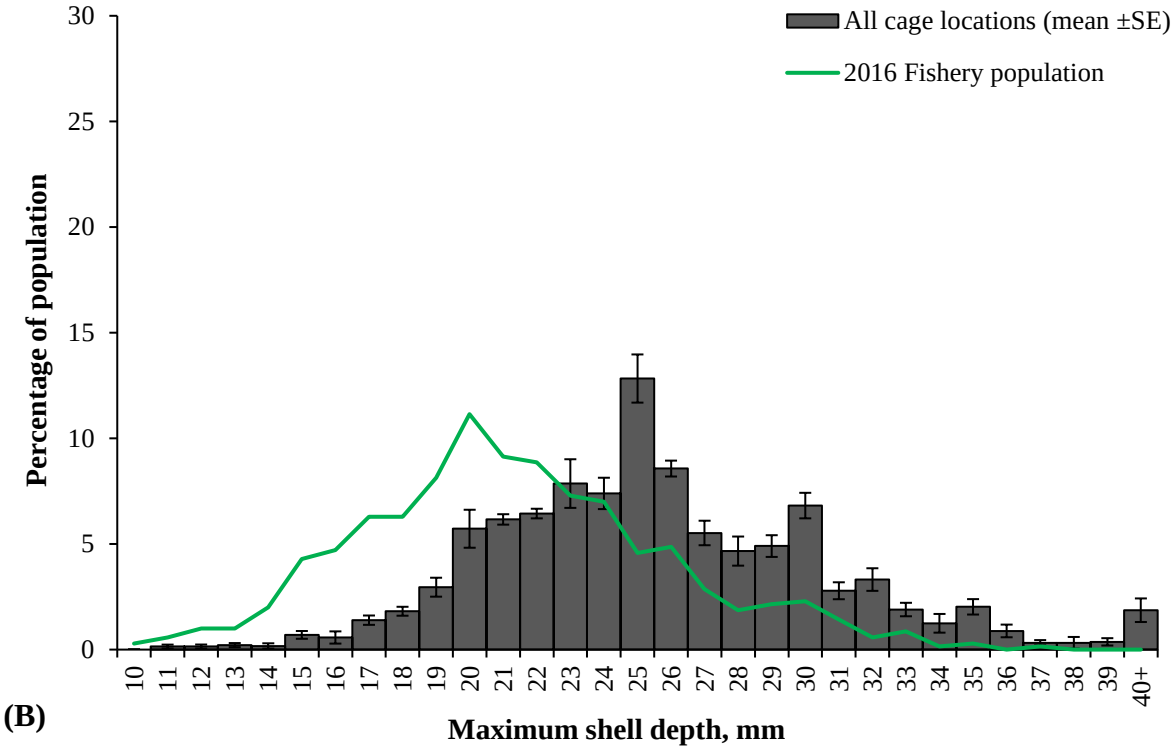


Figure 3.17. (A) Maximum shell depth of deceased individuals collected from each broodstock cage location in comparison to the initial 2016 fishery population used to stock the cages. Data collated into 1 mm arrays. (B) Maximum shell depth of deceased individuals from all broodstock cage locations collected in October 2018 (mean ±SE) in comparison to the initial 2016 fishery population used to stock the cages. Data collated into 1 mm arrays.

3.3.4. Environmental parameters

3.3.4.1. HOBO Pendant® temperature data

Data loggers were deployed for the duration of the two-year study from May 2017 to October / November 2018 at SW, HP and BA. The data logger deployed at PH was not retrieved in May 2018 and was replaced in June 2018. The data logger for the UP population was deployed from May 2017 to March 2018, from April to June 2018 the logger was not deployed but was re-deployed from July to November 2018. The data logger at SP was not retrieved after the 2017 sampling period and was therefore only deployed from December 2107 to November 2018.

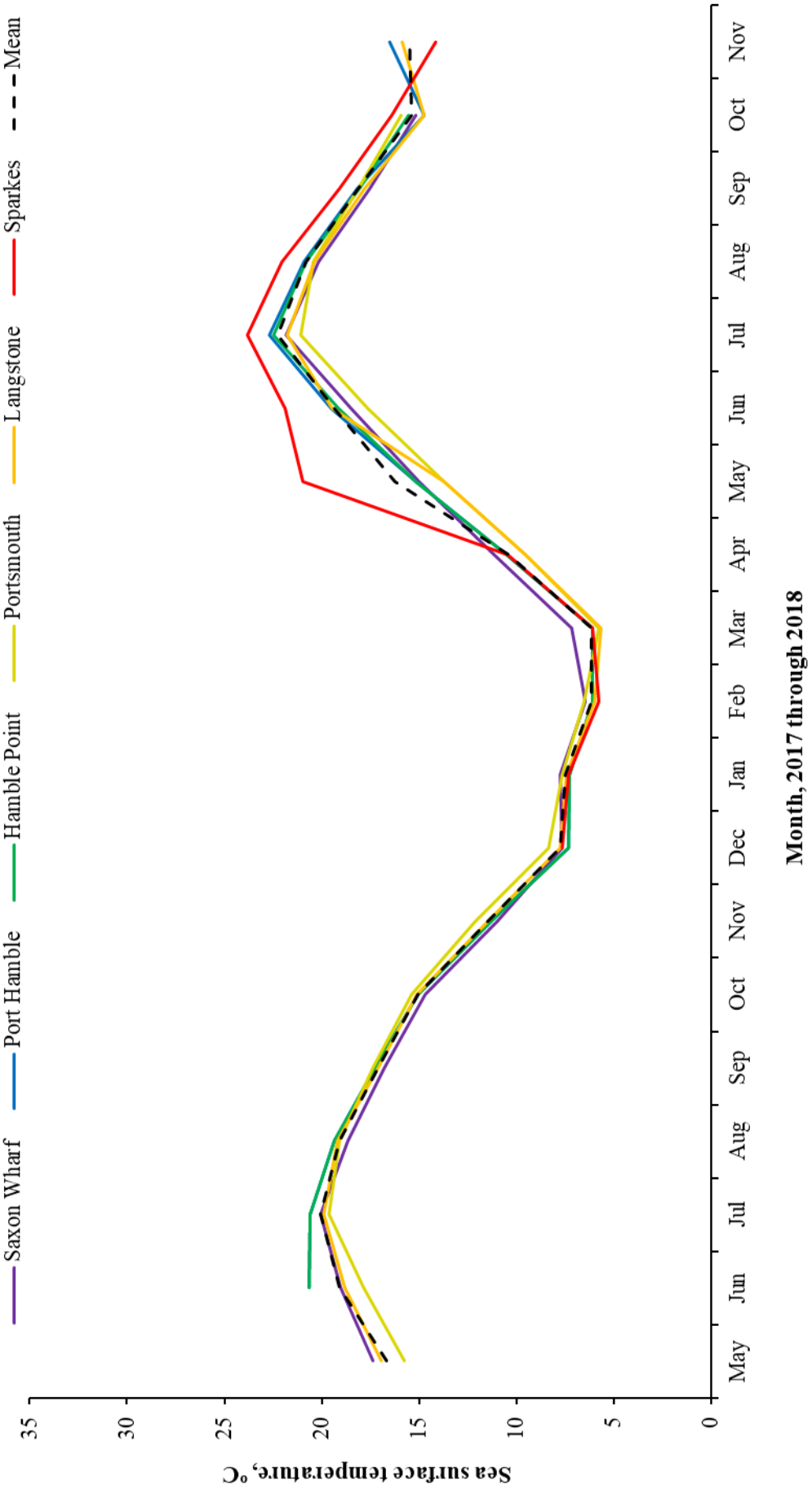
Average sea surface temperature (SST) increased steadily from May to July 2017 where 20°C was recorded for 2017, HP recorded the greatest temperature of 20.6°C during July with the BA location 1°C cooler. From August to December 2017 SST gradually decreased to 7.7°C, with HP experiencing the lowest temperature, 7.3°C, and BA 1°C warmer than this. During this period differences in temperatures between locations was < 1.1°C. This decline in temperature continued into 2018 where the lowest average SST for the whole study were recorded, 6.2 in February and March 2018. After this cooler period SST rose at an average of 4°C / month from March to July where the average SST peaked at 22.3°C. From July to October 2018, SST gradually decreased at an average rate of 2.3°C / month where it reached 15.4°C. A slight increase of 0.1°C in the average SST at three remaining locations, PH, UP and SP was observed from October to November 2018 (Fig. 3.18A).

The highest SST was recorded at UP during May, June, July, August, October and December 2017, 23.8, 25.3, 23.6, 22, 16.9 and 9.7°C, respectively. Equally high recordings were obtained at SW for July and December, and at BA for October. During September the SST was greatest at HP, 20.1°C and at BA during November, 14.2°C. For the first four

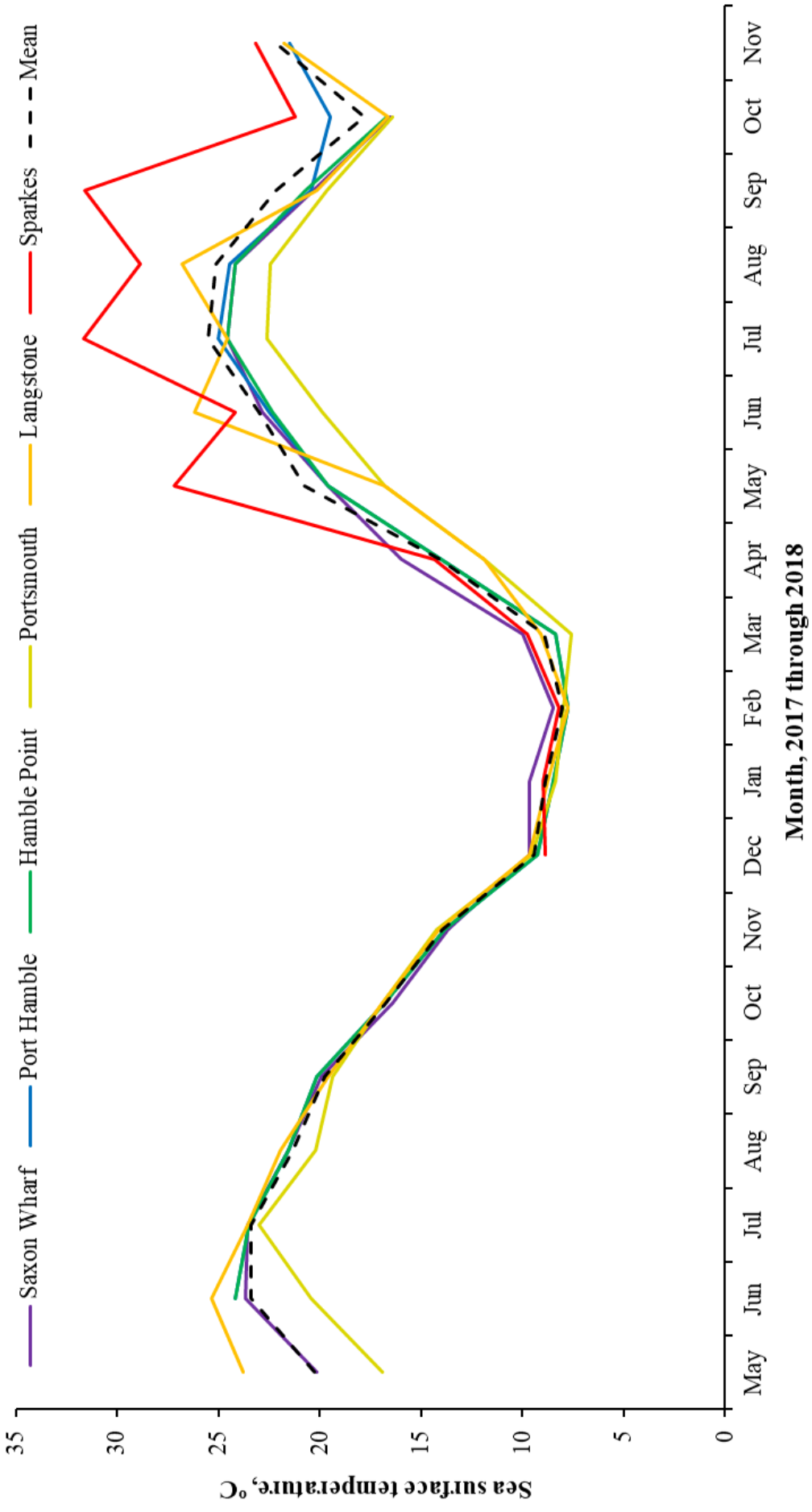
months of 2018 the highest SST was recorded at SW, 9.7, 8.5, 10 and 16°C, respectively. During May, Jul, August, September and October the SST was greatest at SP, 27.2, 31.7, 28.9, 31.6 and 21.2°C, respectively and at UP during June, 26.2°C (Fig. 3.18B).

The minimum SST for May 2017, 14.3°C, was recorded at BA, for June through to December 2017 the lowest SST was recorded at SW, for four of these months the SST was at least 1°C lower than that of the closest recording. From January through to May 2018 the lowest SST was recorded at SP, where temperatures were recorded to be sub-zero in March, -0.2°C. During June and July, the SST was again lowest at SW, 14.1 and 18.5°C, respectively, and then during August and September the lowest recordings were from SP, 13.8 and 5.6°C, respectively. The lowest SST for October 2018 was at PH where the temperature was recorded to have dropped to 3.6°C (Fig. 3.18C).

The maximum SST recordings for SP during May, July and September 2018 were dramatically increased in relation to all other locations, 6.7 - 10.9°C, and the minimum SST recordings for June, August and September 2018 were relatively lower than the other locations, 2.5 - 7.9°C, it is unclear if the section of the cage that the data loggers were attached to at SP became exposed at low tide, during these months, when the cages were on the substratum pushing them above the surface of the water. This may have also occurred at PH in October 2018.


Saxon Wharf
Port Hamble
Hamble Point
Portsmouth
Langstone
Sparkes
Mean
Sea surface temperature, °C
0
5
10
15
20
25
30
35
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Month, 2017 through 2018

(A)


Saxon Wharf
Port Hamble
Hamble Point
Portsmouth
Langstone
Sparkes
Mean
Sea surface temperature, °C
0
5
10
15
20
25
30
35
May
Jun
Jul
Aug
Sep
Oct
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Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Month, 2017 through 2018

(B)

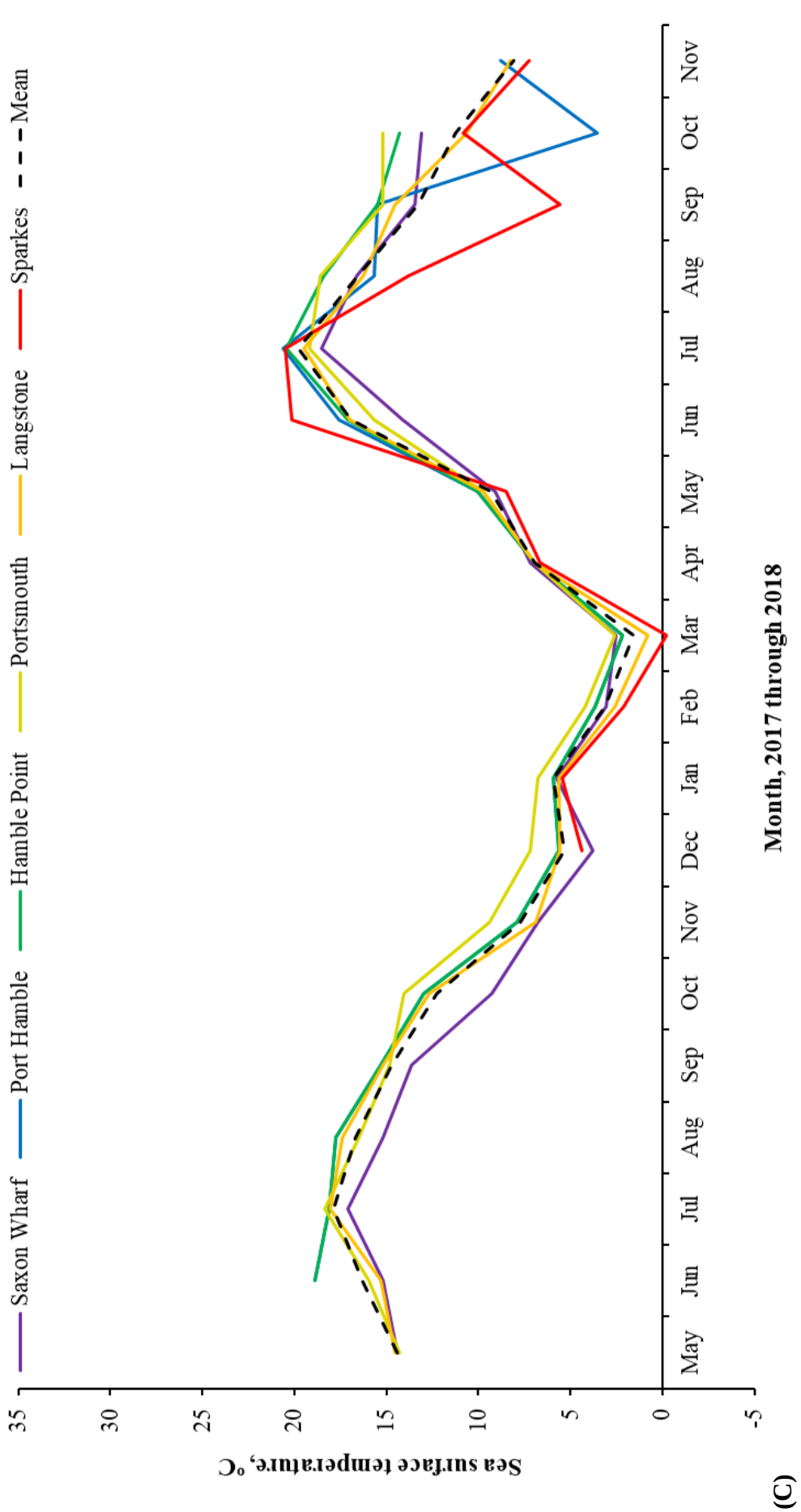


Figure 3.18. (A) Average sea surface temperature, (B) maximum sea surface temperature and (C) minimum sea surface temperature within broodstock cages at Saxon Wharf, Port Hamble, Hamble Point, Portsmouth Harbour, Langstone Harbour and Sparkes Marina during the first (May 2017 - April 2018) and second (May - November 2018) years trials of this study. Data recorded every 15 minutes within the cages using a HOBO Pendant® Temperature/Light 64K Data Logger - UA-002-64 (Onset Computer Corporation, USA).

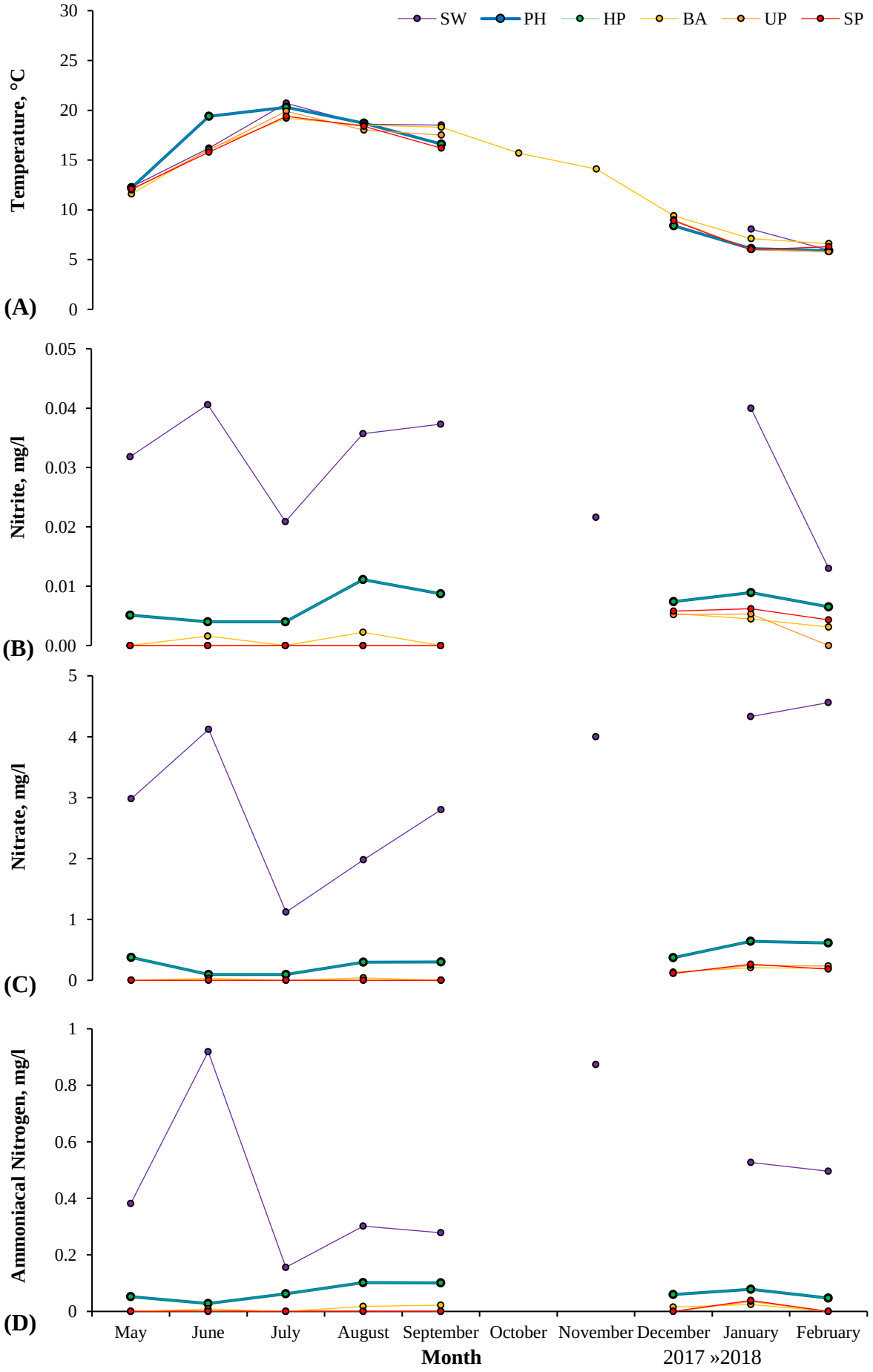
3.3.4.2. Environment agency data

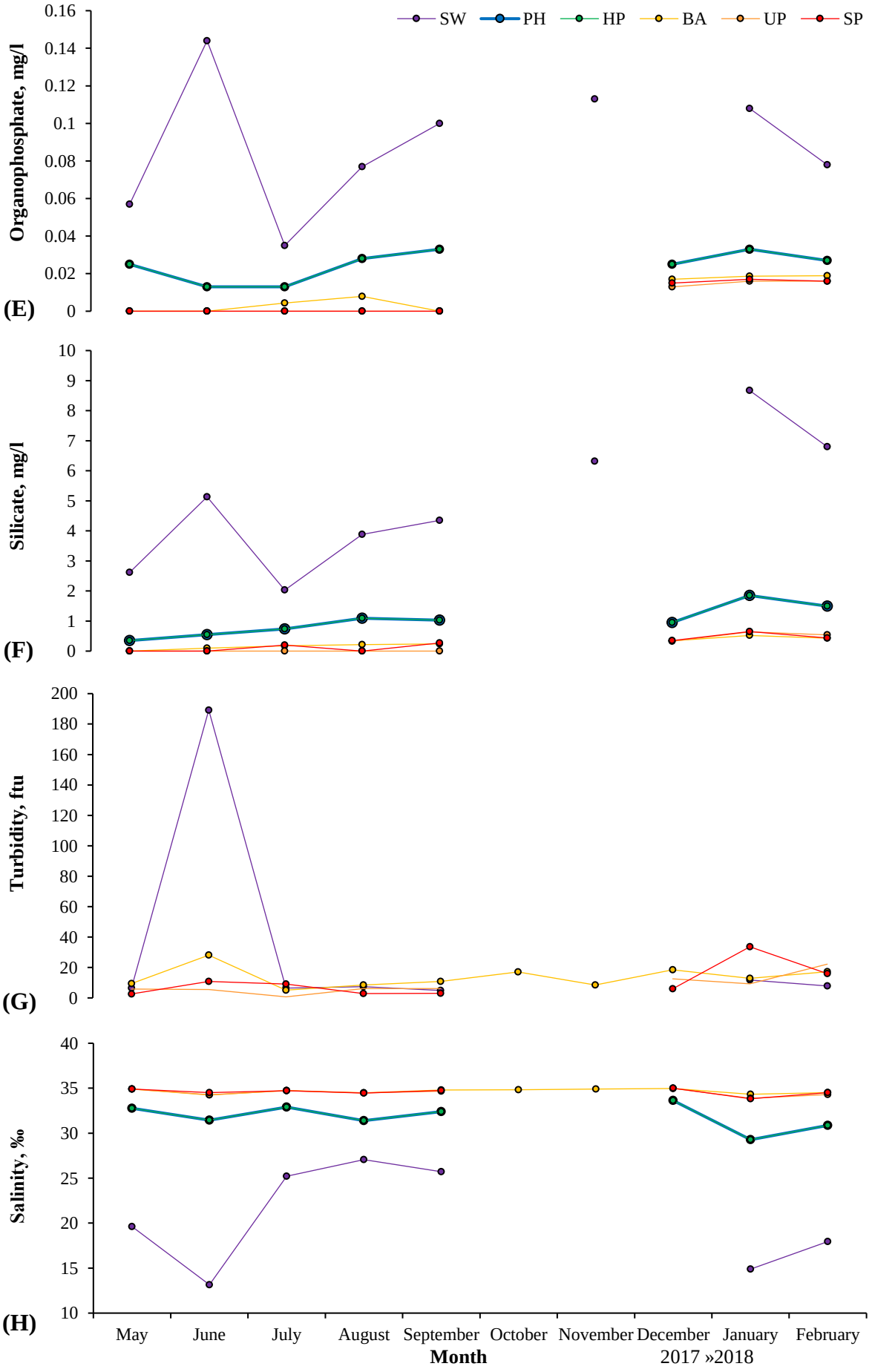
Year 1: May 2017 - April 2018

The monthly temperature followed a similar trend at all locations with an increase from May to July and a steady decrease through the winter months, slight variation between some locations was observed each month ranging from 0.7 - 3.6 °C and averaging 1.6 °C.

Nitrite levels were consistently higher within the populations located in the central Solent area (SW, PH and HP) than those located in the eastern Solent areas (BA, UP, SP) with SW recording the greatest value each month, 0.013 - 0.041 µg/l, followed by the PH and HP populations exposed to a maximum of 0.011 µg/l in August. Populations within the eastern Solent did not exceed 0.006 µg/l. This trend within the populations was also observed with nitrate, ammoniacal nitrogen, organophosphate and silicate recordings. The reverse trend was seen with salinity as the eastern Solent remained between 33.88 - 35 ‰ (34.60 ±0.32 ‰, mean ±SD), the Hamble between 29.28 - 33.63 ‰ (31.84 ±1.39 ‰) and SW was the lowest at 13.15 - 27.06 ‰ (20.51 ±5.56 ‰). Dissolved oxygen levels at PH and HP were consistently lower than at the other locations, 5.53 - 8.96 mg/l (7.34 ±1.21 mg/l).

A sharp peak in turbidity, from 6.5 to 189.2 ftu, at SW in June was matched by peaks in nitrite, nitrate, ammoniacal nitrogen, organophosphate and silicate whilst the lowest salinity was recorded during that period. Following this a peak in chlorophyll (acetone extract) was observed in July when the recordings for all previously mentioned parameters declined drastically. The dissolved oxygen levels also decreased from June through July and into August (Fig. 3.19). A heavy rainfall event cause excessive freshwater input into the system bringing with it run off from the surrounding catchment causing an algal bloom. No abnormal activity as described was observed at any of the other locations where broodstock oysters were situated.


SW
PH
HP
BA
UP
SP
Temperature, °C
(A)
Nitrite, mg/l
(B)
Nitrate, mg/l
(C)
Ammoniacal Nitrogen, mg/l
(D)
May
June
July
August
September
October
November
December
January
February
Month
2017 »2018


SW
PH
HP
BA
UP
SP
Organophosphate, mg/l
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
(E)
Silicate, mg/l
10
9
8
7
6
5
4
3
2
1
0
(F)
Turbidity, ftu
200
180
160
140
120
100
80
60
40
20
0
(G)
Salinity, ‰
40
35
30
25
20
15
10
(H)
May
June
July
August
September
October
November
December
January
February
Month
2017 »2018

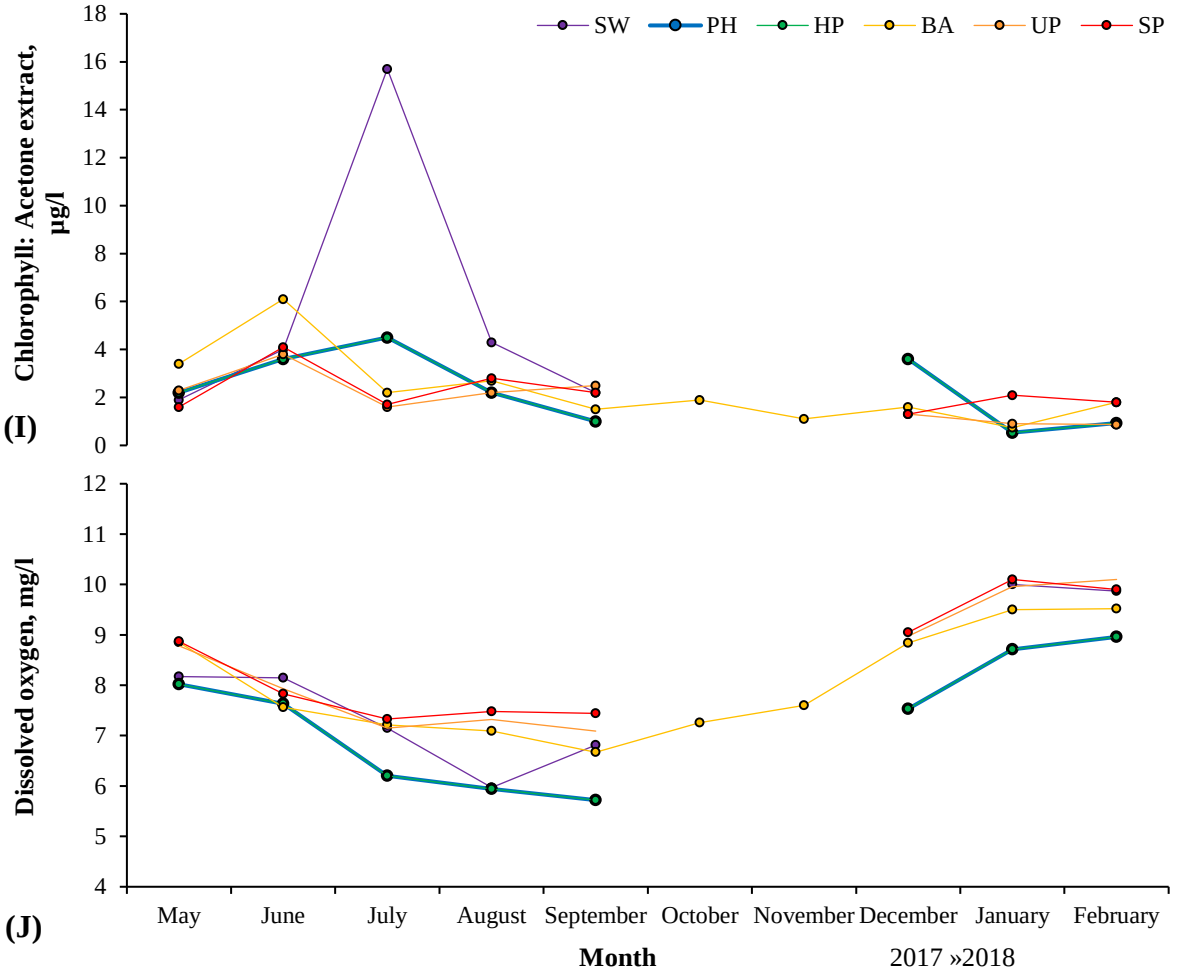


Figure 3.19. Environment Agency (EA) (2019) Water Quality Archive data for (A) temperature (°C), (B) nitrite (mg/l), (C) nitrate (mg/l), (D) ammoniacal nitrogen (mg/l), (E) orthophosphate (mg/l), (F) silicate (mg/l), (G) turbidity (ftu), (H) salinity (‰), (I) chlorophyll : acetone extract (µg/l) and (J) dissolved oxygen (mg/l) from the EA sampling station in closest proximity to the broodstock cages in Saxon Wharf (SW), Port Hamble (PH), Hamble Point (HP), Portsmouth Harbour (BA), Langstone Harbour (UP) and Sparkes Marina (SP). All data was selected where samples were collected at 0.2 m depth from May 2017 to February 2018 in alignment with the first year of broodstock monitoring.

No significant differences were observed in temperature or dissolved oxygen between the central and eastern populations during any month or across all months (Kruskal- Wallis H test, $p > 0.05$). Salinity was significantly lower in the central population during May, July, September and February, and for the total of all months ($p < 0.05$). Nitrite and nitrate recordings were significantly greater in the central population in May, August and September, including the total of all months ($p < 0.05$). Ammonia concentrations were significantly higher in the central population during May, July, August, September and

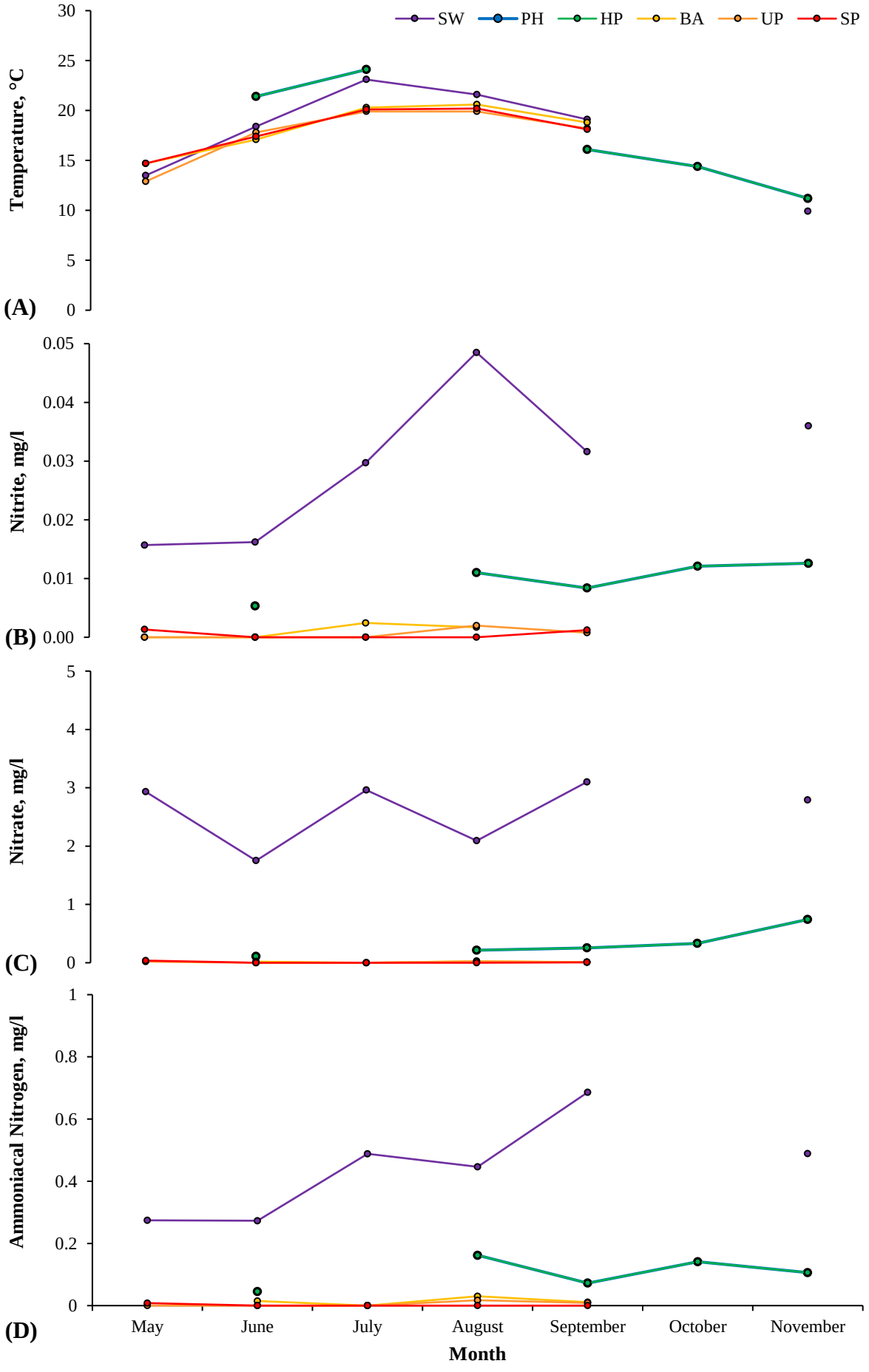
February, as well as for the total of all months ($p < 0.05$). Levels of phosphate (orthophosphate) were significantly greater in the central population during May, August, September and for the total of all months ($p < 0.05$). Silicate was significantly greater in June, July, August and September, and for the total of all months in the central population. During July and December chlorophyll levels were significantly higher within the central population in comparison to the eastern population (Table 3.1).

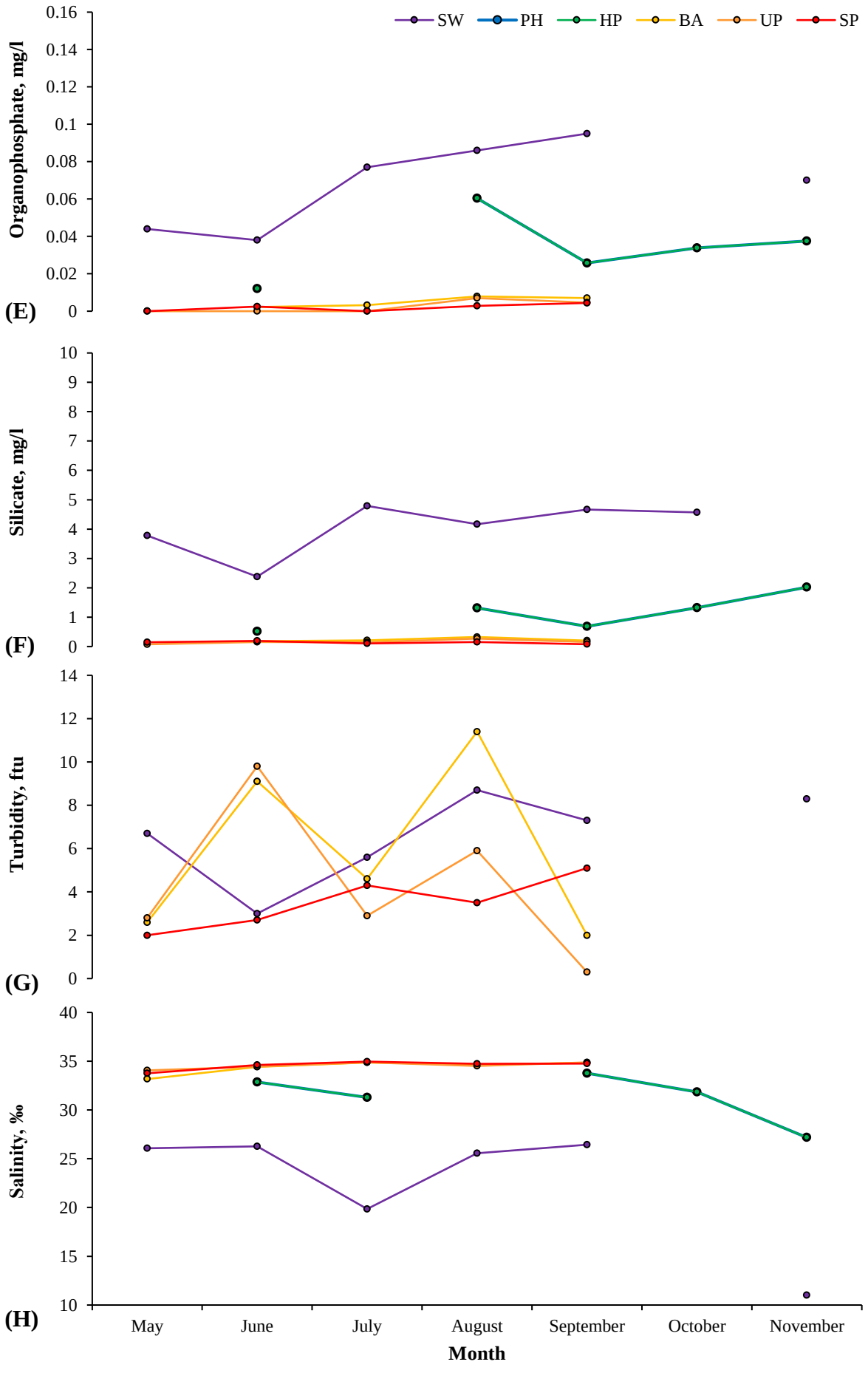
Table 3.1. Comparisons temperature, salinity, turbidity, dissolved oxygen, nitrite, nitrate, ammoniacal nitrogen, orthophosphate, silicate and chlorophyll: acetone extract between the central population (pooled from Saxon Wharf, Port Hamble and Hamble Point) and Eastern population (pooled from Portsmouth Harbour, Langstone Harbour and Sparkes Marina). Statistically significant differences between the two populations for each month and total for all months, are shown with *p* value, absence indicates no significant difference ($p > 0.05$).

	Temp	Salinity	Turb	DO	Nitrite	Nitrate	Amm	Phos	Silicate	Chlor
May		$p = 0.009$	-		$p = 0.036$	$p = 0.005$	$p = 0.020$	$p = 0.013$		
June			-						$p = 0.027$	
July		$p = 0.041$	-				$p = 0.031$		$p = 0.021$	$p = 0.019$
Aug			-		$p = 0.005$	$p = 0.030$	$p = 0.010$	$p = 0.010$	$p = 0.007$	
Sep		$p = 0.021$	-		$p = 0.005$	$p = 0.010$	$p = 0.017$	$p = 0.002$	$p = 0.022$	
Dec			-							$p = 0.046$
Jan			-							
Feb		$p = 0.032$	-				$p = 0.026$			
Total		$p < 0.001$	-		$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	

Year 2: May - November 2018

The environmental conditions observed in the second year of the trial followed a similar trend to the previous year, temperatures increased from May to July and into August before steadily decreasing. Variation was again observed between locations ranging from 0.9 - 4 °C (2 °C mean). As with the first year of the trial, nitrite, nitrate, ammoniacal nitrogen, organophosphate and silicate levels were consistently elevated at SW in comparison to all other locations and salinity between 8 - 16.2 ‰ lower than all locations throughout the trial. Unlike the previous year no correlation with excessive peaks in any of the available parameters was recorded during the time the broodstock cages were deployed (Fig 3.20).


SW
PH
HP
BA
UP
SP
Temperature, °C
30
25
20
15
10
5
0
(A)
Nitrite, mg/l
0.05
0.04
0.03
0.02
0.01
0.00
(B)
Nitrate, mg/l
5
4
3
2
1
0
(C)
Ammoniacal Nitrogen, mg/l
1
0.8
0.6
0.4
0.2
0
(D)
May
June
July
August
September
October
November
Month


SW
PH
HP
BA
UP
SP
Organophosphate, mg/l
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
(E)
Silicate, mg/l
10
9
8
7
6
5
4
3
2
1
0
(F)
Turbidity, ftu
14
12
10
8
6
4
2
0
(G)
Salinity, ‰
40
35
30
25
20
15
10
(H)
May
June
July
August
September
October
November
Month

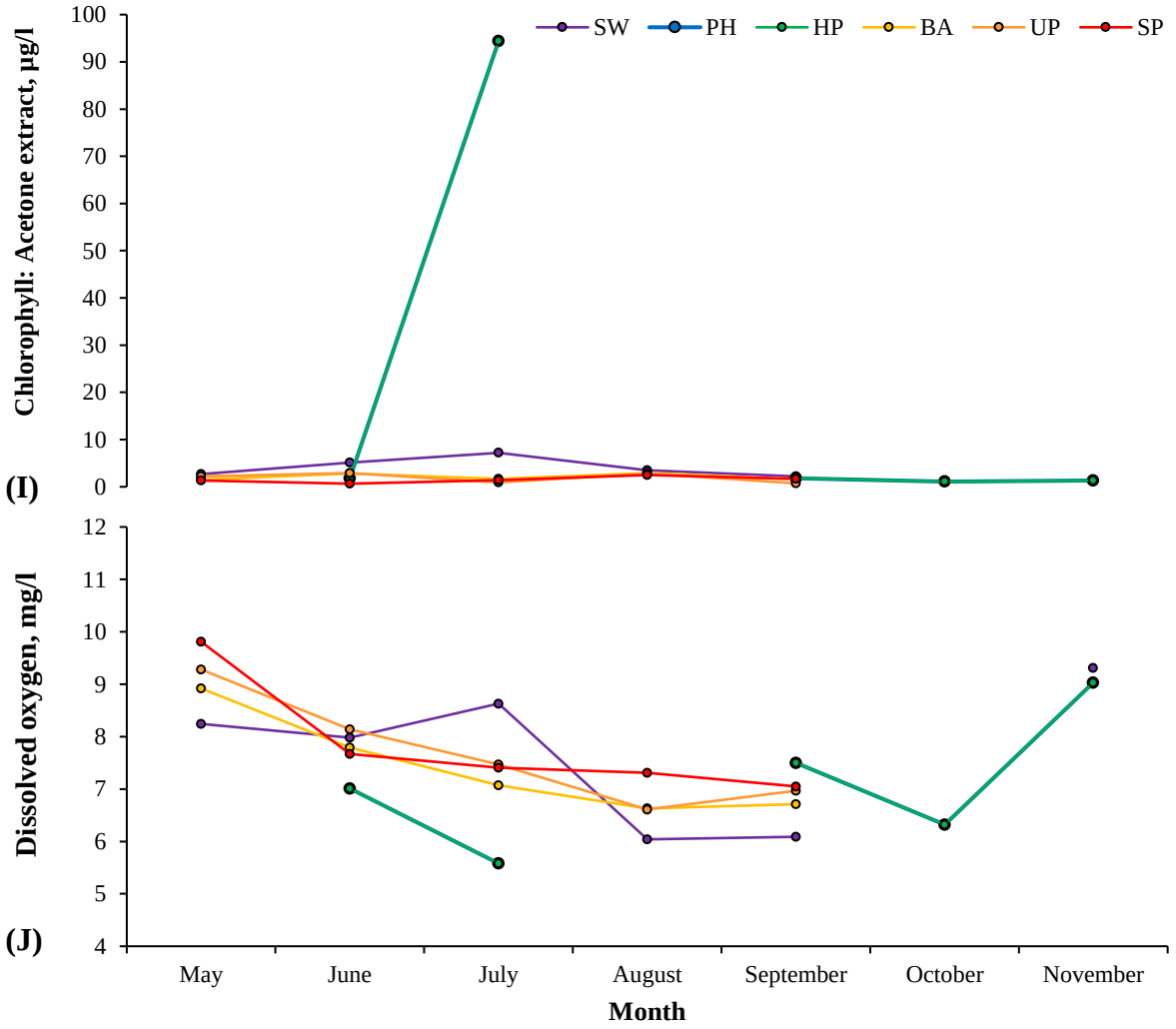


Figure 3.20. Environment Agency (EA) (2019) Water Quality Archive data for (A) temperature (°C), (B) nitrite (mg/l), (C) nitrate (mg/l), (D) ammoniacal nitrogen (mg/l), (E) orthophosphate (mg/l), (F) silicate (mg/l), (G) turbidity (ftu), (H) salinity (‰), (I) chlorophyll : acetone extract (µg/l) and (J) dissolved oxygen (mg/l) from the EA sampling station in closest proximity to the broodstock cages in Saxon Wharf (SW), Port Hamble (PH), Hamble Point (HP), Portsmouth Harbour (BA), Langstone Harbour (UP) and Sparkes Marina (SP). All data was selected where samples were collected at 0.2 m depth from May 2018 to November 2018 in alignment with the second year of broodstock monitoring.

3.3.5. Influence of environmental conditions on mortality and condition

Year 1: May 2017 - April 2018

In total, 90.1 % of the data were explained in the two axes with the high, medium and low mortality forming clusters. Low mortality was associated with turbidity, dissolved oxygen and nutrients (nitrite, nitrate, silicate, ammonia and phosphate). High mortality was associated with temperature and salinity, with no clear association for the medium level of mortality (Fig. 3.21).

Year 2: May - October 2018

The multivariate analysis indicated that 99.6 % of the mortality data were explained by the two axes and that the very high, high and low mortality formed clusters. The environmental parameters nitrite, nitrate, silicate, ammonia and phosphate were strongly associated with the occurrence of very high mortality whilst the occurrence of high mortality was associated with dissolved oxygen and turbidity. Low mortality was shown to be associated highly with salinity (Fig. 3.22).

The multivariate analysis indicated that 75.7 % of the 2018 condition index data were explained by the two axes. The data formed clusters for the designated groups 0 - 2, 2 - 4, 4 - 6 and 6 +. The higher condition (6 +) was associated with nutrients (nitrite, nitrate, ammonia, and phosphate). Condition indices between 2 - 4 were associated with temperature and salinity. The condition indices ranging from 4 - 6 were not shown to be associated with any of the environmental parameters (Fig. 3.23).

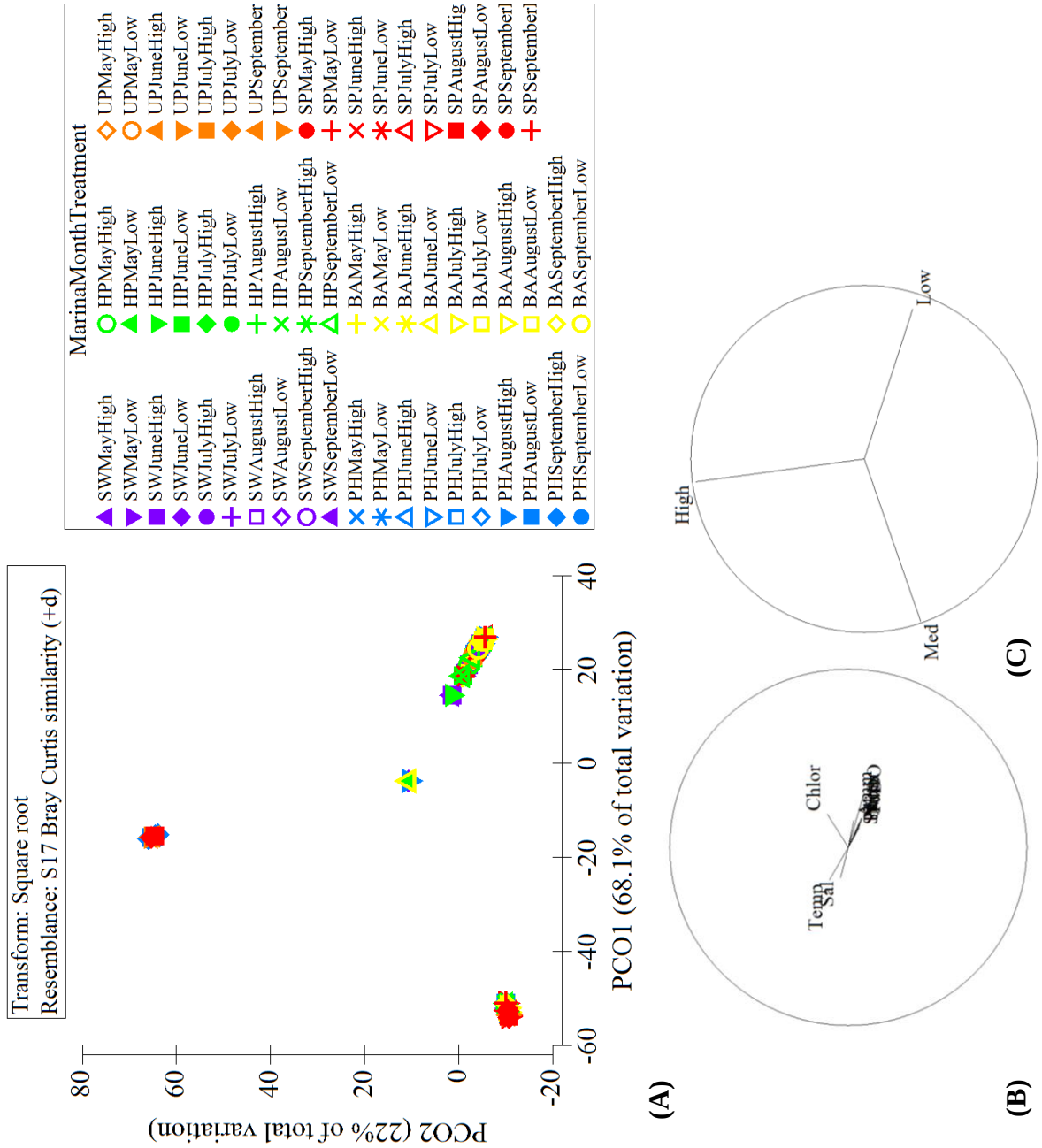
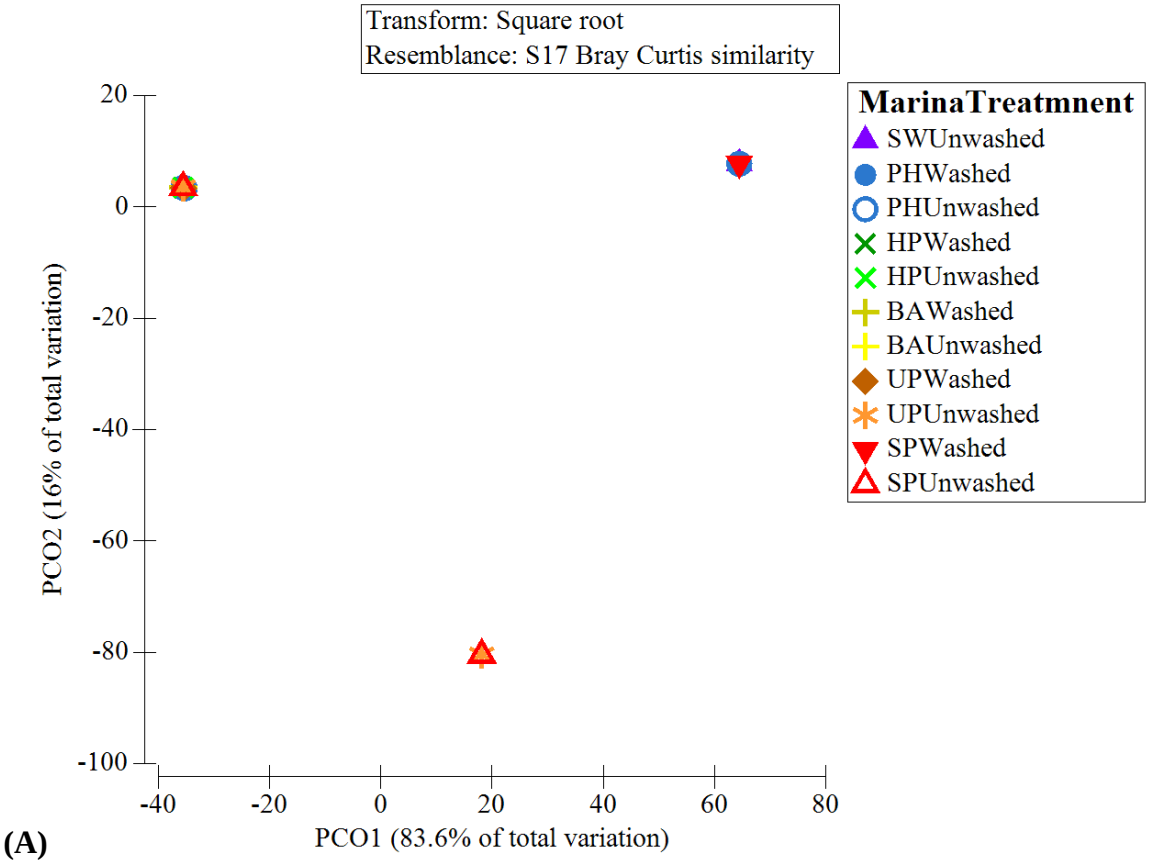
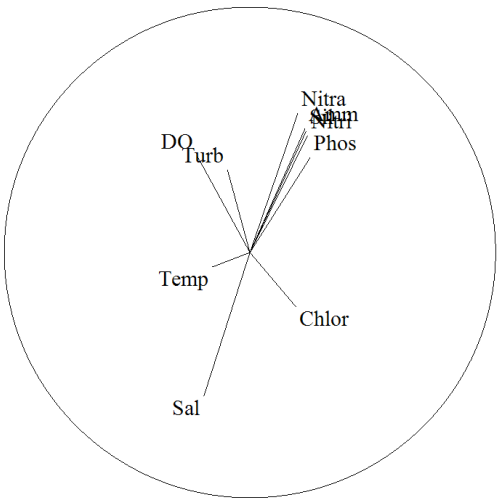


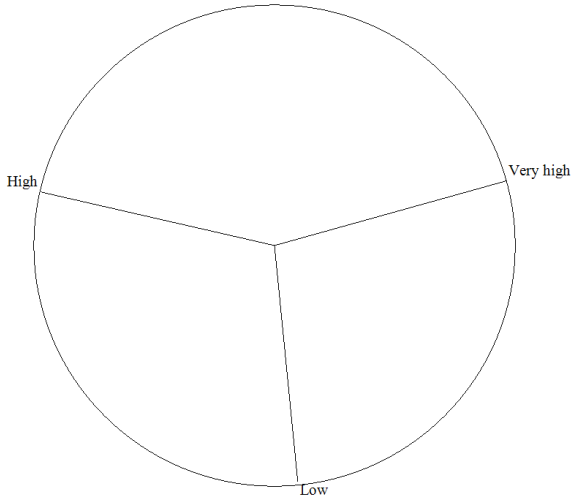
Figure 3.21. Principle coordinates analysis (PCO) expressed as ordinations. (A) The variation of percentage broodstock mortality during the 2017 trial (May 2017 - September 2017), from all locations in full and half densities, in relation to; (B) available Environment agency water quality data from the most geographically relevant sampling location for each location. (C) Values were grouped into low (0 - 20 %), medium (20 - 40 %) and high (40 % +), the strongest relationship explaining the scatter of mortality is correlated with temperature (Temp) and salinity (Sal) for the high mortality, with turbidity (Turb), dissolved oxygen (DO) and nutrients (nitrite (Nitri), nitrate (Nitra), ammonia (Amm), silicate (Sil) and phosphate (Phos)) associated with low mortality. Codes: SW - Saxon Wharf; PH - Port Hamble, HP - Hamble Point; BA - Portsmouth Harbour; UP - Langstone Harbour; SP - Sparkes Marina; Washed - pressure-washed; Unwashed - not pressure-washed.



(A)



(B)



(C)

Figure 3.22. Principle coordinates analysis (PCO) expressed as ordinations. (A) The variation of percentage broodstock mortality at the end of the 2018 trial, from all locations with pressure-washed and unwashed oysters, in relation to; (B) available Environment agency water quality data from the most geographically relevant sampling location for each location. (C) Values were grouped into low (0 - 50 %), high (50 - 80 %) and very high (80 % +), the strongest relationship explaining the scatter of mortality is correlated with nutrients (nitrite (Nitri), nitrate (Nitra), ammonia (Amm), silicate (Sil) and phosphate (Phos)) for the very high mortality, dissolved oxygen (DO) and turbidity (Turb) for the high mortality and salinity (Sal) for the very low mortality values. Codes: SW - Saxon Wharf; PH - Port Hamble, HP - Hamble Point; BA - Portsmouth Harbour; UP - Langstone Harbour; SP - Sparkes Marina; Washed - pressure-washed; Unwashed - not pressure-washed.

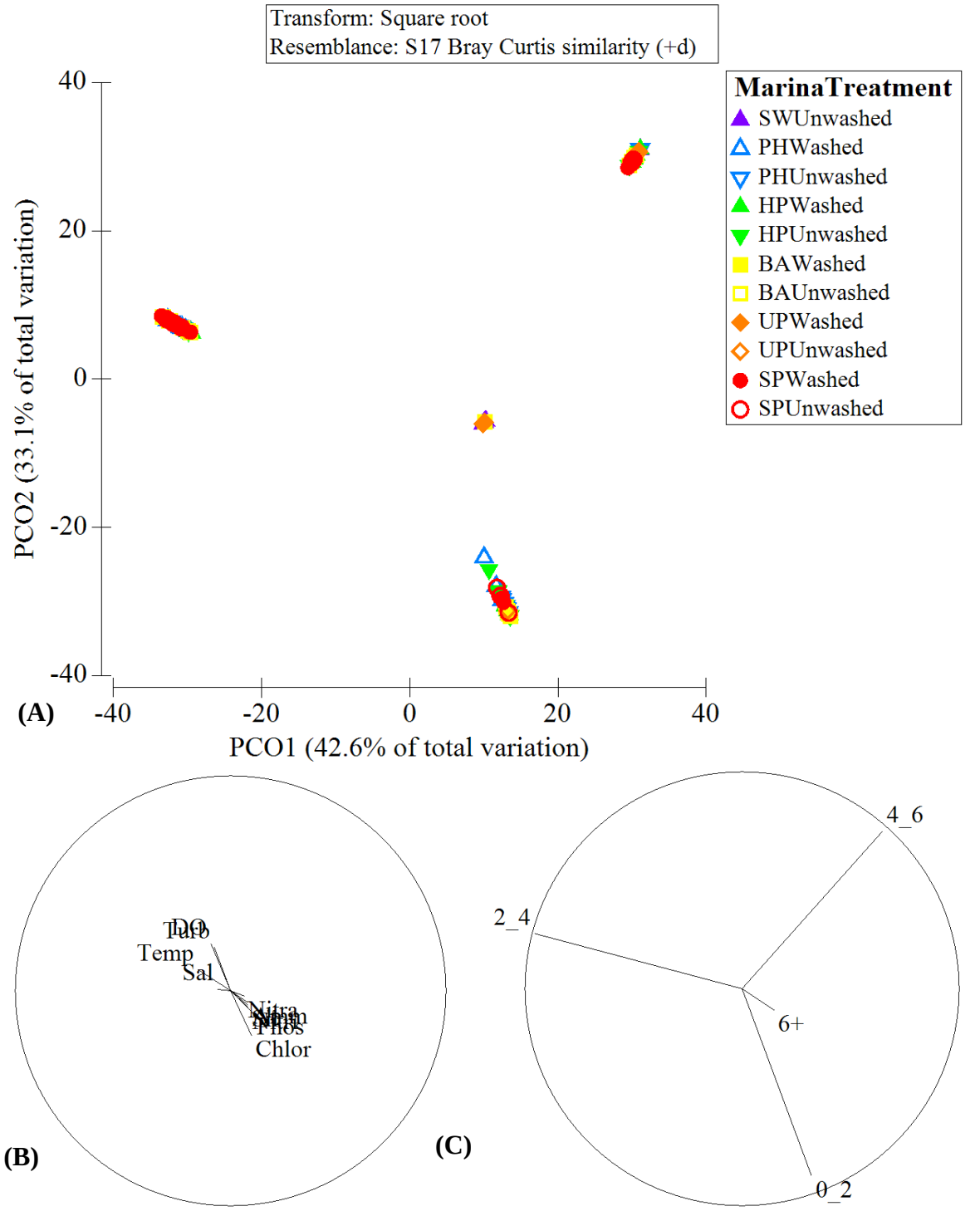


Figure 3.23. Principle coordinates analysis (PCO) expressed as ordinations. (A) The variation of broodstock condition indices (CI) at the end of the 2018 trial, from all locations with pressure-washed and unwashed oysters, in relation to; (B) available Environment agency data from the most geographically relevant sampling location for each location, the strongest relationship explaining the scatter of CI is correlated with nutrients (nitrite (Nitri), nitrate (Nitra), ammonia, (Amm) and phosphorous (Phos)) for CI of 6 +, temperature (Temp) and salinity (Sal) for the medium CI, chlorophyll for the high CI and dissolved oxygen and turbidity for the very high CI values. (C) Condition index values were grouped into 0 - 2, 2 - 4, 4 - 6 and 6 + for analysis.

3.4. Discussion

Protection and conditioning of broodstock populations is critical for the recovery of *O. edulis* populations across Europe. Many oyster aquaculture systems are focused on optimising the growth of juveniles to enable them to reach marketable size within limited time periods. The focus of this section of the study was to determine the efficacy of prolonged survival within mature oyster populations, housed within a similar, but novel system, in an attempt to allow for multiple reproductive cycles to occur, thus increasing larval output to the benthos. Previous studies have demonstrated improved physiological performance of *O. edulis* on elevated reef structures (80 cm above seabed) (Sawusdee *et al.*, 2015), greater oyster densities, survival and reef complexity within *Crassostrea virginica* populations raised more than 30 cm from the sea bed (Colden *et al.*, 2017) and that sedimentation is influenced by reef orientation in relation to tidal currents (Colden *et al.*, 2016). By suspending oysters in cages at the surface it was anticipated that similar responses would have been observed with the additional benefit that fishing pressure would be completely removed, predation levels would be reduced, and food availability would be increased. Limitations associated with accessing field sites on marine vessels and / or with divers are also removed by utilising the marina environments in the manner of this study.

This issue surrounding inaccessibility was highlighted by the only location, in Langstone Harbour (UP), that required the use of a vessel to access. The inability to assess and restock the cages at this location skewed the data the following month in a manner that it then appeared to be significantly greater than the other locations, which may not have been the case if the same sampling protocol could be conducted during August 2017 and January 2018. Despite this, the low levels of mortality, < 8.1 %, observed during the initial months were extremely encouraging, with this trend continuing at Saxon Wharf (SW) throughout 2017 as average mortality remained < 14 %. The increase in mortality in early 2018, also

experienced by all other populations, was likely to reflect the increase in duration, from one to two months, between sampling time points.

The increase in mortality in experienced during July 2017 could, in part, be attributed to the peak in sea surface temperature coinciding with increased brooding activity in June, see 4.3.1, thus, allocation of energetic resources to gonadal growth that would inevitably induce a lower tolerance to environmental stressors. This is supported by the lowest occurrence of brooding activity and similarly low mortality within the SW population. In contrast, all other locations experienced the highest occurrence of brooding activity during June 2017, leaving those individuals energetically compromised and as a result experienced a drastic increase in mortality over the ensuing month.

This however does not explain the significant difference in mortality observed between Port Hamble (PH) and Hamble Point (HP) populations in July, 36.9 % compared with 20.8 %. These populations are located along the same river system and are < 1 km apart from one another, suggesting that an acute localised event occurred at PH increasing mortality. The cause is not known but could have been as a result of a fuel spillage or effluent discharge from vessels as it is unlikely that environmental conditions fluctuated substantially between the two marinas to an extent that would cause such an increase in mortality, but this cannot be completely ruled out.

A similar occurrence was observed at Sparkes Marina (SP) in August 2017, mortality at all other locations, with the exception of UP for reasons previously mentioned, was between 11 - 17 %, however, mortality at SP was over double the average of the other locations at 31.3 %. This elevated mortality continued into September where again, with the exception of the UP population, the cages at SP experience greater levels of mortality than all other locations that remained at a similar level to the previous month. Fouling was particularly intense at this location during the summer months and was often dominated by

tunicates (pers. obs., Harris-Scott *et al.* Under Review) which may have increased competition for resources and limited the flow of water through the broodstock cages, it is not clear if this would have resulted in such elevated mortality as fouling was also prominent at other locations.

The extremely high mortality in Portsmouth Harbour (BA) during January 2018, continuing into April 2018 also provides cause for concern and it is again likely that an acute anthropogenic factor is the causative agent in this instance. Unfortunately, it was not within the remit or capacity of this study to monitor contamination levels, with regards to heavy metals, polychlorinated biphenyls (PCB), polycyclic aromatic hydrocarbons (PAHs), organophosphates and others. Being situated in a heavily industrialised setting, it is possible that level of contaminants at BA exceeded the tolerance threshold of *O. edulis* in the area, diesel or oil spills also pose a threat in the area with multiple reports of such events throughout the study (A. Munro, pers. comm.).

The elevated mortality at many of the locations in April 2018 is likely to reflect both the increase in sampling duration and environmental conditions associated with the cold front dubbed the "Beast from the East", despite these conditions mortality was not as elevated as would be expected as seen with many benthic species around the UK. However, this could provide an explanation for the mass mortality event observed at SW at the inception of the second year's trial. With a greater freshwater input, the effects of excessive snowfall and low temperatures were likely exacerbated at this location. It has been suggested that *O. edulis* is a stenohaline species with a tolerance of salinities between 25 - 37 ‰ (Korringa, 1941), the Environment Agency salinity recordings at SW were at the lower limit of, or below, this threshold for periods of normal weather conditions throughout 2017 and 2018. Unfortunately, likely due to the extreme nature of weather conditions experienced, the environmental parameters were not available for that period of time, but it could be

suggested that the prolonged and extreme decline in salinity that ensued excessive snow and rainfall during this weather event, exceeded the tolerances of the oysters situated in this particular marina, affecting biological processes and inducing mortality.

This potential impact of salinity could also provide an explanation for the increased, albeit non-significant in most populations, mortality observed within the pressure-washed oysters during the 2018 trial. This is supported by the treatment of the UP population, which, due to logistical limitations, were treated with pressurised seawater from a deck hose during all but one month, where pressurised freshwater was used. Mortality within the washed oysters at this location was lower than all other locations which received freshwater cleaning treatment throughout the duration of the experiment. However, mortality was also lowest within the unwashed population at this location suggesting that proximity and adaptation to environmental conditions of oyster source location may have also influenced survival. This was also likely to be influenced by the replenishing of one cage from oysters house at the Institute of Marine Sciences.

This proximity to source population location may also explain the trend observed with condition indices of the different broodstock populations. The UP population, within the same body of water as the source population, had a significantly increased condition index compared with those in Portsmouth Harbour, Chichester Harbour and the River Hamble. It would also appear that those oysters that tolerated the pressure-washing treatment, at all locations, were able to feed more readily with the reduced competition from fouling organisms as the condition index values were higher, but not significantly, in all but one population compared with unwashed oysters. Unexpectedly, the ten surviving oysters in the population experiencing the greatest mortality at SW recorded the greatest condition index, indicating that the oysters at this location were in fact healthy and that an acute event caused the mass mortality seen during May and June 2018. This mortality even was unusual as the

temperature recordings obtained from within the cages does not reveal any trend that should be cause for concern in relation to the other marinas and no mass mortality was observed within the feral population of *Crassostrea gigas* or established population of *Mytilus edulis* in the marina.

Alongside environmental conditions, the manner in which the oysters were arranged also influenced mortality, with the impact of density highlighted with monthly variations between full and half-density populations. Despite no statistical difference, the mortality within half-density populations was 1.1 - 4 % lower during four of the six months in 2017. Mortality within full-density populations increased within the micro-reef positions within the bottom section of the cages, it is likely that this is due to the increased surface area of settlement substrata provided by a greater quantity of oysters allowing for excessive fouling to adhere in these populations. The micro-reef structures were therefore unable to support the weight in this manner and the feeding capacity of those oysters within the middle and, especially, bottom units would have been suppressed or at least required a greater energetic output to force the valve apart to allow feeding to take place. This trend was not seen within the half-density populations and as would be expect, due to the slight reduction of environmental stressors, mortality decreased marginally in the middle and bottom units. Further issues with the micro-reef structure were made apparent by the disparity between the size of the original fishery population used to stock the cages and the size at which mortality was occurring most frequently. The finding suggest that the micro-reef structures are not suitable for medium to large broodstock oysters with regards to shell length (> 65 mm) and depth (> 20 mm), also this size of oyster may have been approaching the end of their natural expected lifespan.

The current structural design of the broodstock cages would not allow for large quantities of broodstock to be sustained for prolonged periods, however, with modifications

to the internal housing mechanisms and alternative management approaches it is likely that high mortality during the summer months could be reduced to a point whereby reproductively viable densities of oysters could be sustained. Reducing the stocking density and increasing the quantity of cages used to stock a similar number of broodstock, would allow restoration projects to mitigate against the effects of acute localised pollution events. Selecting oysters of a smaller size would also prolong the duration spent within the system and allow for the occurrence of a greater number of reproductive cycles. By assessing environmental parameters in areas intended for restoration practices such as that used in this study, the suitability of particular water bodies can be assessed prior to deployment of broodstock, avoiding unnecessary mortality whilst maximising conditioning for larval output.

Chapter 4

The Efficacy of Suspended Broodstock Cages as a Restoration Strategy - Reproduction

4.1. Introduction

Monitoring for, and recording the occurrence of, any reproductive activity is vital to *Ostreae edulis* restoration activities, many of which include the long-term goal of self-sustaining populations, whereby larvae produced by the adult population and other adult populations in relatively close proximity will allow for multiple year classes to establish and seed the future generations. Understanding the reproductive capacity of the adult broodstock oysters used in the cages for this study was vital to not only understanding the likely number of larvae that would be produced as a result of the intense stocking densities, but also in understanding the timing and duration of brooding. This, in-turn, allows for appropriate timing of settlement substrate deployment. This chapter reports on the occurrence of larval brooding within suspended cages across the Solent, the number of larvae contained within each brood in relation to adult size and location, and any settlement of spat observed with and without broodstock cages. These differences between populations are reported on from oysters that were obtained from the same source location in Langstone Harbour. It is hoped that the results present will also inform policy making decisions regarding the parameters used to select for the minimum landing size of fishery oysters, if and when the fishery becomes active once more.

4.2. Methods

4.2.1. Broodstock sex ratio

During the May - October 2017 broodstock cage sampling period, sex ratio and gonadal development of caged oysters was monitored. During each month, at each of the six sampling locations, three randomly selected live oysters were removed from each full-density and each half-density cage that were monitored at that point in time. These individuals were placed into individually labelled / sealed plastic bags, put on ice and returned to the laboratory. Upon return to the laboratory, any epibionts were removed and morphometric measurements were recorded for all individuals, as in 2.2.3. Once complete, the oysters were then shucked by carefully severing the hinge ligament with an oyster knife, any larvae contained within the pallial cavity were carefully extracted into 30 ml polypropylene universal containers (Fisher Scientific), see 4.2.2. Once any larvae were removed, a 5 mm cross section of the visceral mass were collected for further histological analysis following the protocol of Howard and Smith (1983). Tissue sections were fully submerged in Bouin's fixative (CellPath, UK) within a 30 ml polypropylene universal container (Fisher Scientific) for a minimum of 24 h, after which the fluid was replaced with 70 % ethanol for a minimum of 24 h. The solution was then replaced with fresh 70 % ethanol. These samples were preserved for future analysis that was not able to be conducted within the time frame of this study.

4.2.2. Brooding period and broodstock fecundity

4.2.2.1. Year 1: 2017

Oysters were collected monthly between May and October 2017 as in 4.2.1 and once shucked were examined for presence of larvae and classified into white, grey and black "sick" stages (Helm *et al*., 2004) (Fig. 4.1). It should be noted that the colouration to the naked eye is not a completely reliable guide to the exact developmental stage of larvae (Cole, 1941). The larvae from any individuals that were brooding were rinsed with filtered seawater into a 30 ml polypropylene universal container (Fisher Scientific), in the rare case into two appropriately labelled containers. Larvae were allowed to settle at the base of the container before any excess fluid, including the filtered seawater, was removed using an automated pipette before preservation in 98 % ethanol, prior to analysis. Fecundity was measured as the number of larvae brooded per oyster.

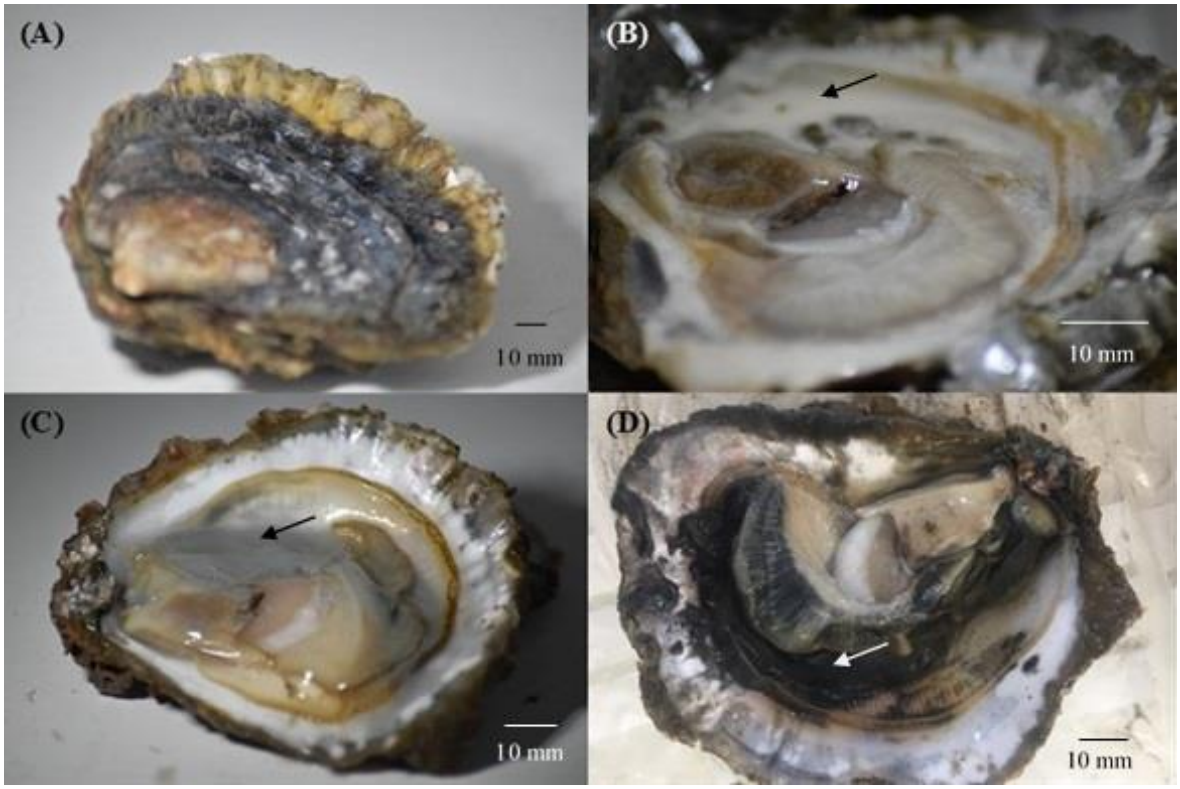


Figure 4.1. (A) Brooding oyster prior to shucking, brooding oysters containing larvae referred to as (B) white "sick", (C) grey "sick" and (D) black "sick" stages of development. Larvae can be seen within the pallial cavity in and around the gill and mantle structures.

The day prior to the intended analysis date, samples were drained, then rinsed with and stored in saline solution, made to 31 psu using Tropic Marin® salt. Total sample volume was made up to 50 ml in a falcon tube. Initial calibration of the MultisizerTM 3 Coulter counter (Beckman Coulter Life Sciences, USA) with 2000 µm aperture (Meritics, UK) using a 100 µm monodisperse polystyrene microsphere-size standard (Sigma-Aldrich) in 200 ml 0.2 µm Tropic Marin® salt electrolyte solution (31 psu, 3% w/v), resulted in an unacceptable level of noise. Excess noised indicated that the flow rate through the aperture was too great, and that the addition of glycerol was required to increase the viscosity of the electrolyte. Samples were therefore preserved using 4% paraformaldehyde in PBS (Alfa Aesar™) and stored at 4 °C until sufficient quantities of glycerol were sourced. Subsequent electrolyte solution was mixed with glycerol (Fisher Scientific) in a 6:4 ratio and filtered through a 0.2 µm filter, allowing for successful calibration. Background noise level was as ≤ 86.28 µm, thus the minimum size threshold was set to 86.28 µm and the counting range set to 86.28-400 µm with the flow rate calculated to be 4.325 ml/s.

Samples were then mixed vigorously within the falcon tubes; a 2 ml aliquot of the sample was removed from the center of the tube and pipetted into a beaker. 198 ml of electrolyte was then added to this aliquot to distribute the larvae evenly around the solution before running the sample for 20 s (86.5 ml analytical volume). The Coulter counter was briefly flushed with electrolyte solution for 4 s before analysis began to remove any remnants of the previous sample from the aperture. This procedure was repeated in triplicate for each sample. Particle Counts were recorded for each 2 ml sample and Mean Particle Counts were calculated from the triplicate measurements. Total Larval Counts for each oyster were then calculated from the Mean Particle Counts as follows:

$$\textit{Mean Particle Count} \times (200/86.5) = L_2 \rightarrow L_2 \times 25 = L_{50}$$

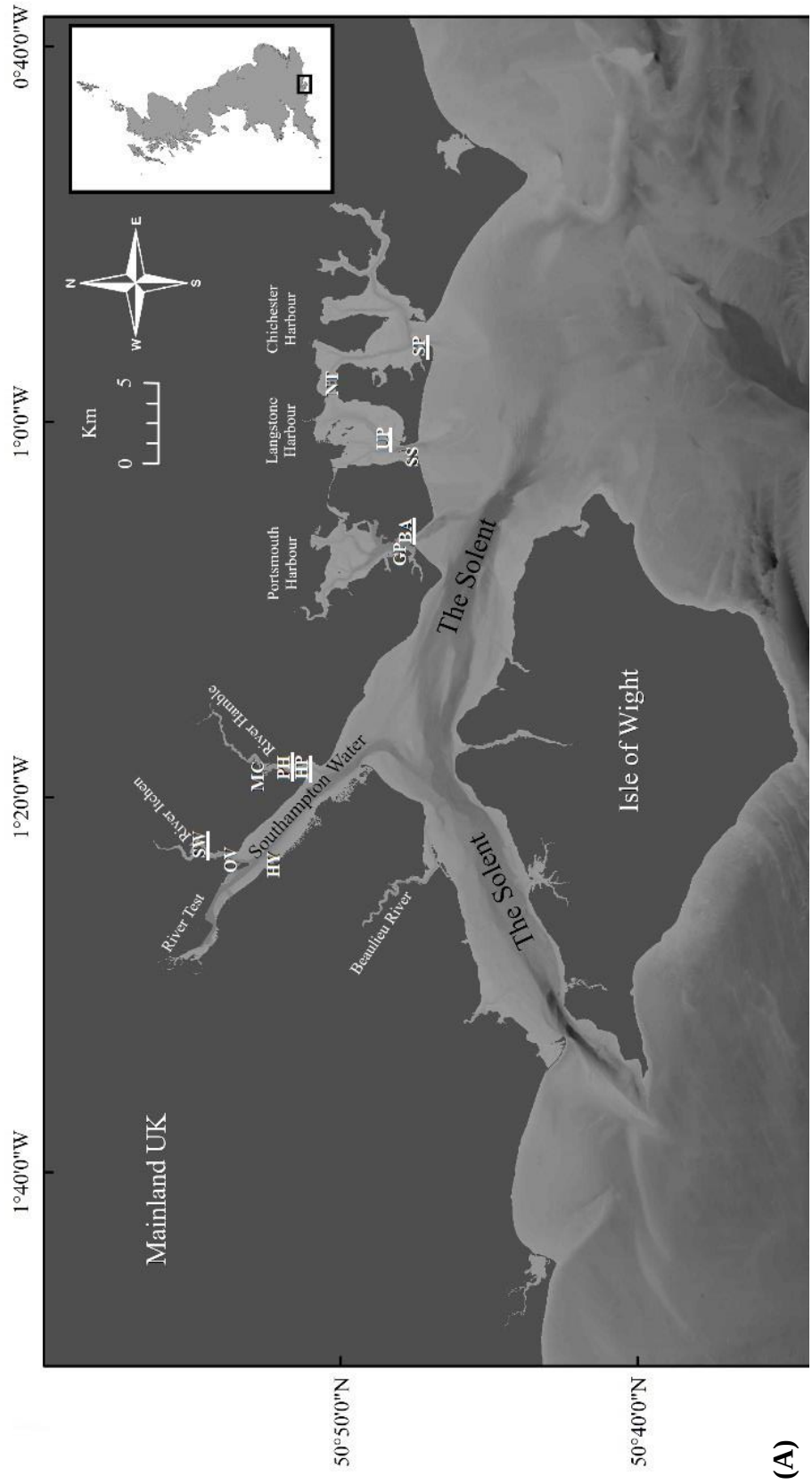
Where L_2 = Larvae per 2 ml sample diluted in 198 ml electrolyte, and L_{50} = Total Larval Count in original 50 ml volume. Particles size was also produced by the Multisizer™ 3. A small proportion of each brood was also used to determine presence of pathogenic protozoans, see Chapter 5.

4.2.2.2. Year 2: 2018

To minimise the impact of sacrificial sampling on the broodstock populations, the second years sampling was conducted by removing individuals from the cage system and submerging them in a solution of magnesium chloride ($MgCl_2$) at a concentration of 30 ppt contained within a 750 ml container. Observations were made between Portsmouth, Langstone and Chichester Harbours for the 2018 season. Observations were only conducted from the end of July onward. Oysters sampled were submerged in the solution for a maximum of three hours, if after this period they were unresponsive there were marked as "did not open" and excluded from the trial. Those that were susceptible to the anaesthetic effects of the $MgCl_2$ were observed for the presence of larvae within the pallial cavity. Larvae that were present were rinsed into the 750 ml container and transported to the laboratory where they were passed over a 40 μm mesh sieve before being transferred to a 30 ml polypropylene universal container, preserved with 70 % ethanol and stored at 4 °C. Further analysis was conducted as in 4.2.2.1. A proportion of the larvae from each sample were also used to determine disease prevalence, see Chapter 5.

4.2.3. Larval settlement

To assess the success of larval dispersal and settlement from the broodstock cage systems, settlement plates were constructed from roofing tiles (Burton Roofing Merchants Limited, Redland Plain Tile Antique Red). Settlement plates were deployed across the Solent in the six marinas where broodstock cage populations were located at Saxon Wharf (SW), Port Hamble (PH), Hamble Point (HP), Portsmouth Harbour (BA), Langstone Harbour (UP) and Sparkes Marina (SP). Six marinas locations, where broodstock cages were absent but, that were in close proximity were also selected and settlement plates were deployed at these locations of Hythe Marina Village (HY - waiting pontoon), Ocean Village (OV), Mercury Marina (MC), Gosport Marina (GP), Southsea Marina (SS - waiting pontoon) and Northney Marina (NT) (Fig. 4.2). In marina locations where broodstock cages were present settlement plates were suspended in as close proximity as was logistically possible but did not extend more than 10 m from the closest broodstock cage. Deployment was conducted in July 2017 and July 2018, shortly after the main peak in spawning was believed to occur in the Solent (Kamphausen *et al*., 2011) allowing for the development of a biofilm to encourage settlement (Walne, 1974). Removal of the settlement plates occurred approximately six months after deployment for the 2017 plates (January 2018) and ten months after deployment for the 2018 plates (May 2019), allowing for larval settlement and development to a size whereby they could be observed with the naked eye in amongst the anticipated fouling community.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Southampton Water
River Hamble
River Itchen
River Test
Beaulieu River
Portsmouth Harbour
Langstone Harbour
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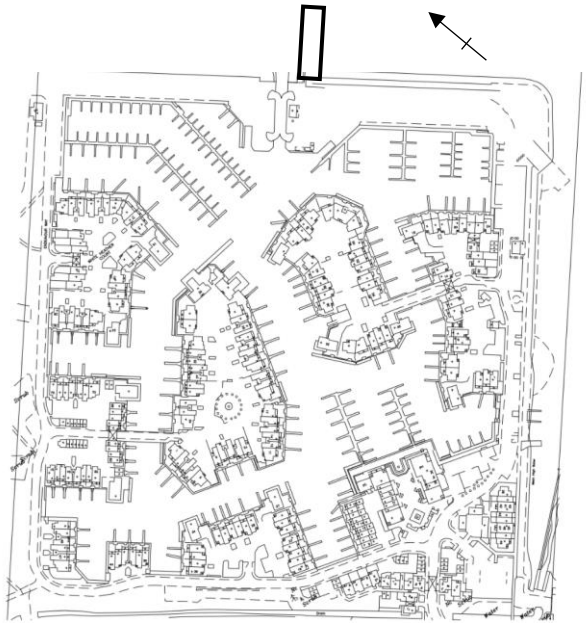
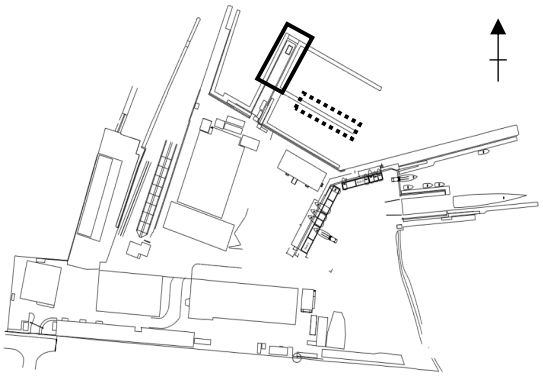
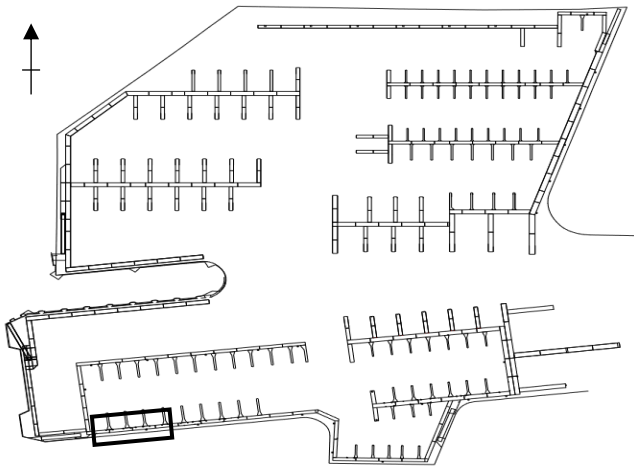


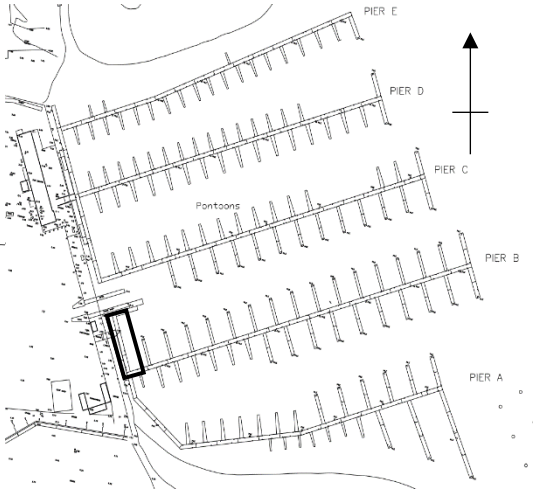
(B) Hythe Marina Village



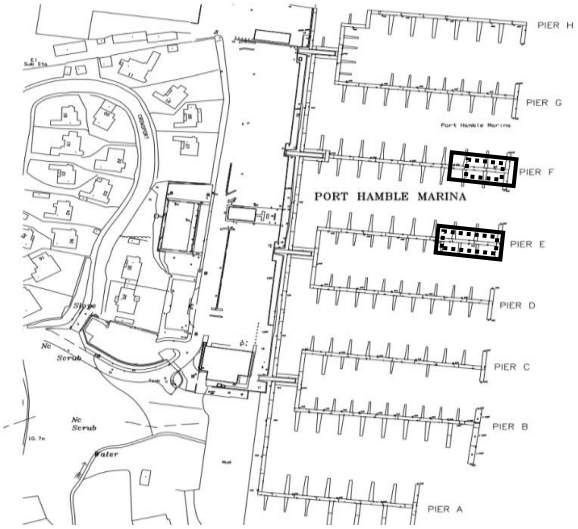
(C) Saxon Wharf



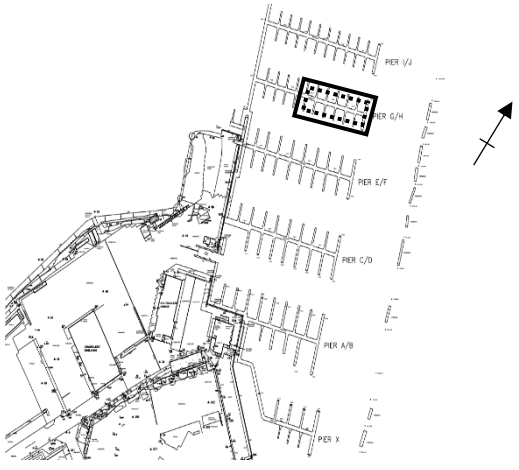
(D) Ocean Village Marina



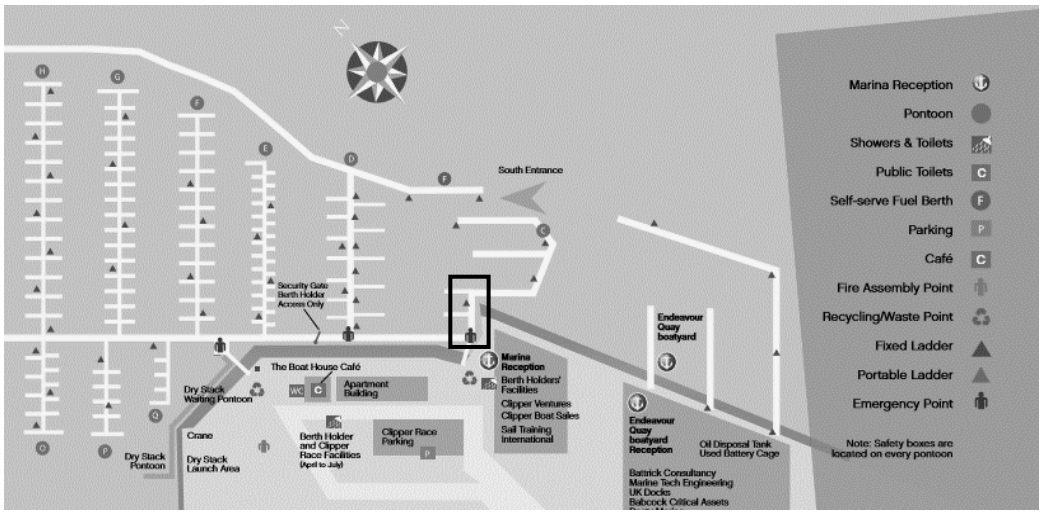
(E) Mercury Marina



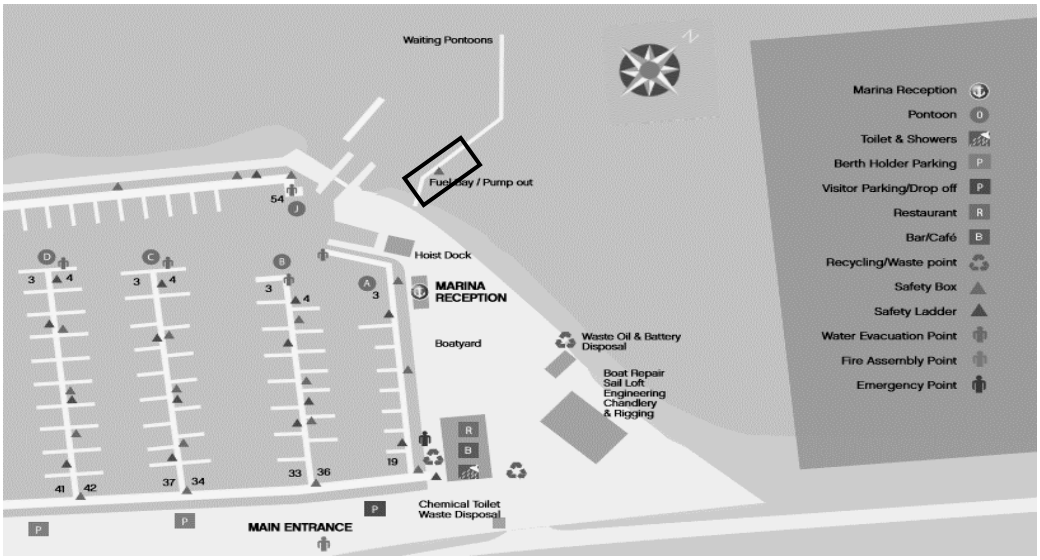
(F) Port Hamble Marina



(G) Hamble Point Marina

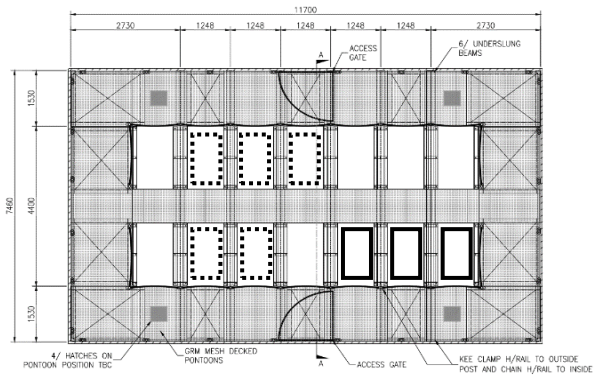


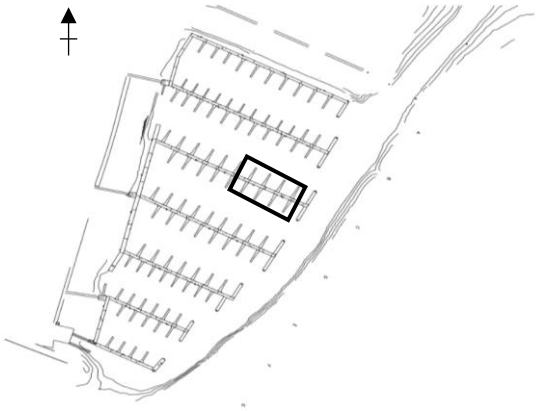
(H) Gosport Marina



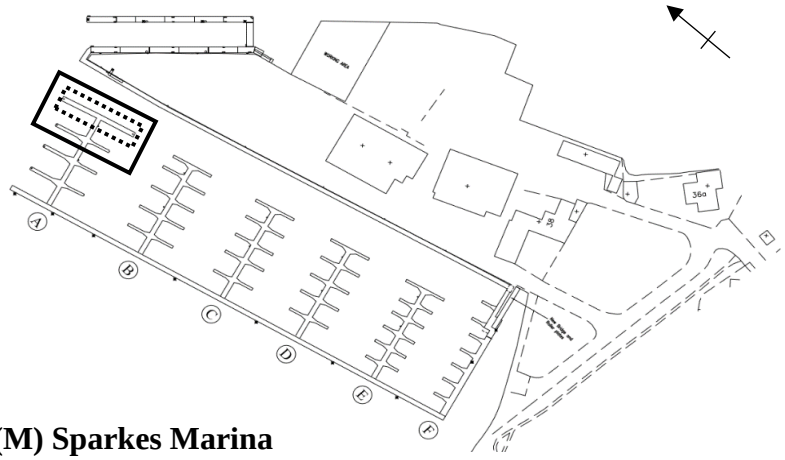
(J) Southsea Marina

(I) Camber Dock, Portsmouth Harbour





(K) University of Portsmouth Research Platform (L) Northney Marina



(M) Sparkes Marina

Figure 4.2. (A) Sampling locations in relation to the wider Solent. Areas underlined indicating marina locations that contained suspended broodstock cage populations, shown with dashed boxes, and areas not underlined indicate marina locations that did not contain suspended broodstock cage populations. Locations of settlement plates, show within solid boxes, on floating structures across the Solent including (B) Hythe Marina Village waiting pontoon, (C) Ocean Village Marina, (D) Saxon Wharf, (E) Mercury Marina, (F) Port Hamble Marina, (G) Hamble Point Marina, (H) Gosport Marina, (I) Camber Dock, (J) Southsea Marina waiting pontoon, (K) University of Portsmouth research platform, (L) Northney Marina and (M) Sparkes Marina. Locations of settlement plates are denoted within the solid boxes at all marinas and are shown in relation to locations of *Ostrea edulis* broodstock cages in D, F, G, I, K and M.

Coordinates are as follows:

Site	Latitude	Longitude
B	50° 52.557'N	1° 23.944'W
C	50° 53.660'N	1° 23.522'W
D	50° 54.786'N	1° 22.752'W
E	50° 52.240'N	1° 18.724'W
F	50° 51.652'N + 50° 51.617'N	1° 18.698'W + 1° 18.695'W
G	50° 51.202'N	1° 18.686'W
H	50° 47.791'N	1° 7.033'W
I	50° 47.544'N	1° 6.429'W
J	50° 47.543'N	1° 2.032'W
K	50° 47.543'N	1° 1.345'W
L	50° 49.992'N	0° 57.968'W
M	50° 47.191'N	0° 56.596'W

Two orientations were used for this section of the study following previous observations of *O. edulis* preferences towards underside of horizontal environments, even in the aquaria setting (Cole and Knight Jones, 1939) and recent reports of *Ostrea angasi* settling on the underside of pontoons in Port Phillip Bay, Victoria, Australia (B. Cleveland, pers. comm.). Half of the tiles allocated to each marina (n = 3 / marina / year) were hung in a vertical orientation by first using a 5 mm drill bit, followed by an 8 mm drill bit to accommodate 8 mm rope. The tiles were then suspended by threading 1 m lengths of polypropylene rope through each hole that were then tied off to available cleats or alternative solid structure at each marina. Each tile had both a smooth surface on one side and a rough surface on the other, settlement was noted for these respective surfaces (Fig. 4.3).

The other half of the tiles allocated to each marina (n = 3 per marina, per year) were hung in a horizontal position (as previously described). To achieve the same hanging depth as the vertical tiles a 2 m length of rope was threaded through each of the holes at either end of the tile. Two ends of the rope from one end of the tile were tied off to a cleat or alternative structure and then the other two ends of the rope were adjusted and manoeuvred to allow the tile hang horizontally.

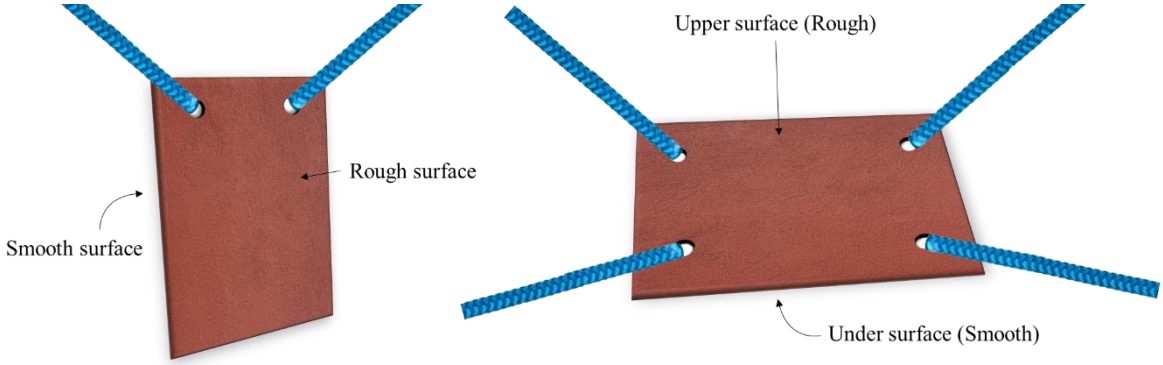


Figure 4.3. The two orientations of settlement plates used in the settlement trial in marinas both with and without broodstock cage, left vertical, right horizontal. Three replicates of each orientation were deployed in each marina location in 2017 and a further three replicates in 2018. Diagram is not to scale, tile dimensions: 27 x 16.5 x 1 cm.

Larger epibionts were removed post-deployment in the laboratory and settlement of *O. edulis* and *C. gigas* was recorded for each of the tiles and each of the surfaces. For the vertical tiles settlement was recorded on smoother or rougher surface and for horizontal tiles settlement was recorded as topside (rougher) or underside (smoother).

4.2.4. Relative abundances of oyster and limpet larvae in the water column

Plankton tows were conducted in the entrance to Portsmouth, Langstone and Chichester Harbours bi-weekly from May to September 2017. Samples were collected by towing a plankton net with a 20 µm mesh and 30 cm diameter in a sinusoidal manner at one knot for five minutes, with three replicates collected per location at each sampling interval. Due to time and logistical constraints the analysis of the samples collected in the plankton tows could not be completed within the remit of this study, but further analysis aims to determine the relative abundances of *O. edulis*, *C. gigas* and *C. fornicata* larvae over this duration. This will allow interactions between larval abundances and successful recruitment to be assessed and related to benthic populations of mature populations.

4.2.5. Environmental parameters

Environmental conditions were monitored as in 3.2.6.

4.2.6. Statistical data analysis

Data analysis for this section of the study was conducted in R (R Core Team, 2017), IBM® SPSS® Statistics 25 (IBM Analytics, USA) or PRIMER-e v. 6 (Clarke and Gorley, 2006) as shown in Table 4.1. For analysis conducted in R, plots of randomized quantile residuals, and residuals against fitted values were checked for normality, autocorrelation and homoscedasticity. The best model for each region was selected based on minimizing the Akaike Information Criterion (AIC) score, and was carried forward. The best model was selected based on minimising the AIC score, including only those variables that were significant to $p < 0.05$ according to step-wise model selection.

Table 4.1. Statistical analysis conducted in this section of the study using R, SPSS or PRIMER-e v. 6.

Section	Data	Software	Model/Test
Brooding adults	Occurrence of brooding vs density	SPSS	Mann-Whitney U
Brooding adults	Environmental parameters	Primer-e	PCO
Broodstock fecundity	Marina location, Month (as factor), Density, Adult length, Larval count	R	GLM poisson distribution
Broodstock fecundity	Larval quantities vs adult morphometrics	SPSS	Pearson correlation
Broodstock fecundity	Larval counts - Environmental influence	Primer-e	PCO
Larval settlement	Spat settlement - between locations		Kruskal-Wallis H
Larval settlement	Spat settlement - between species and settlement orientation		Kruskal-Wallis H

4.3. Results

4.3.1. Broodstock brooding period

4.3.1.1. Total population

The observed brooding activity across all locations occurred from May 2017 - September 2017 with a total of 20.5 % of the population confirmed to be brooding larvae during that period. When calculated to the whole population scale, using the number of live oysters at the beginning of each month, remaining from the initial stocking density of 10,000 oysters, then the total number of individuals that can be estimated to be brooding would be 958.

Peak brooding activity occurred in June with 6, 4 and 0.7 % of the population containing white, grey and black “sick”, respectively and was the only month where observations of black “sick” were made throughout the season. July was the only other month where grey “sick” was observed (1.1 %) and 2.8 % of the population contained white “sick”, the second highest occurrence. White “sick” was observed in 2.8, 2.2 and 0.9 % of the total population in May, August and September, respectively (Fig. 4.4A).

The monitoring for brooding activity for the 2018 season could only be conducted from the end of July onward. Brooding was only detected at Sparkes Marina with two individuals that were observed brooding grey “sick” veliger larvae and one individual continuing white “sick” veliger larvae at the end of July, with no further observations of brooding activity throughout the remaining season (Fig. 4.4B).

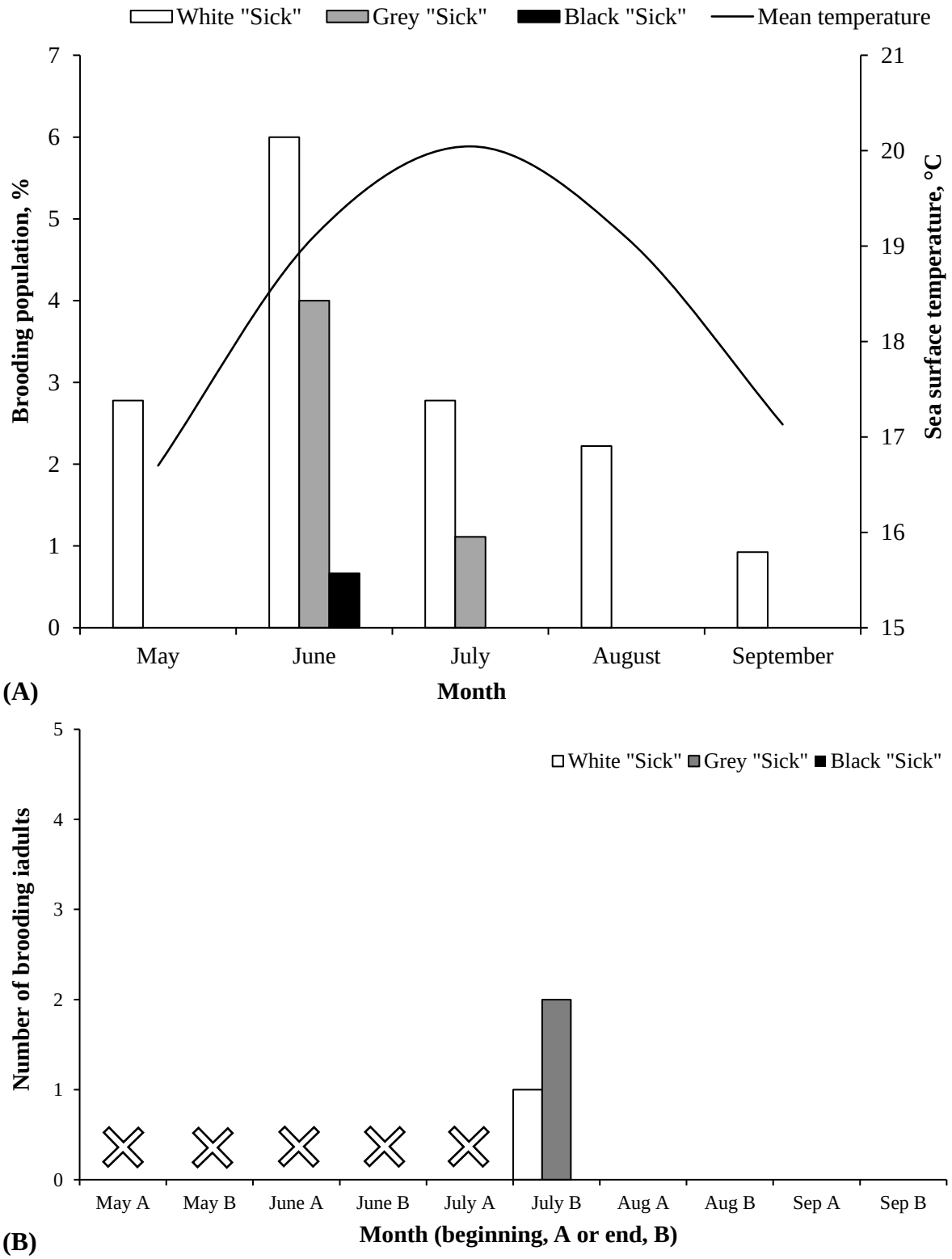


Figure 4.4. (A) The percentage of the total population from all six locations brooding larvae in the white, grey or black "sick" stages of larval development during May (n = 180), June (n = 150), July (n = 180), August (n = 90) and September (n = 108) 2017 with sea surface temperature calculated from all available locations, and; (B) the number of individuals brooding from eastern Solent populations of Portsmouth, Langstone and Chichester Harbours during July (n = 30), August (n = 30) and September (n = 30) 2018. For the 2018 season data X denotes durations that were not monitored.

4.3.1.3. Saxon Wharf

Throughout the 2017 brooding period two individuals were observed brooding white "sick" at Saxon Wharf, one during June and one during July, representing 3.3 % of the monitored population for each of those months (Fig. 4.5A). Of all the sites this was the fewest number of brooding oysters observed throughout the brooding period.

4.3.1.4. Port Hamble

A total of four individuals were observed brooding white "sick" at Port Hamble during the 2017 brooding period, two during July (6.7 %), one in August (5.6 %) and one in September (5.6 %) which represents the latest activity in the season (Fig. 4.5B). Observations were not made in June.

4.3.1.5. Hamble Point

Three individuals were observed brooding larvae at Hamble point during the 2017 brooding period, two individuals in July (6.7 %), one white "sick" and one grey "sick" brood. The third was observed in August, containing white "sick" (3.3 %) (Fig. 4.5C).

4.3.1.6. Portsmouth Harbour

Across the brooding period in 2017 four individuals were observed brooding larvae, three of these during June (10 %) and one in July (3.3 %). Two of the individuals in June contained white "sick" and the other contained the only recorded occurrence of black "sick" for all locations. The individual in July contained white "sick" (3.3 %) (Fig. 4.5D).

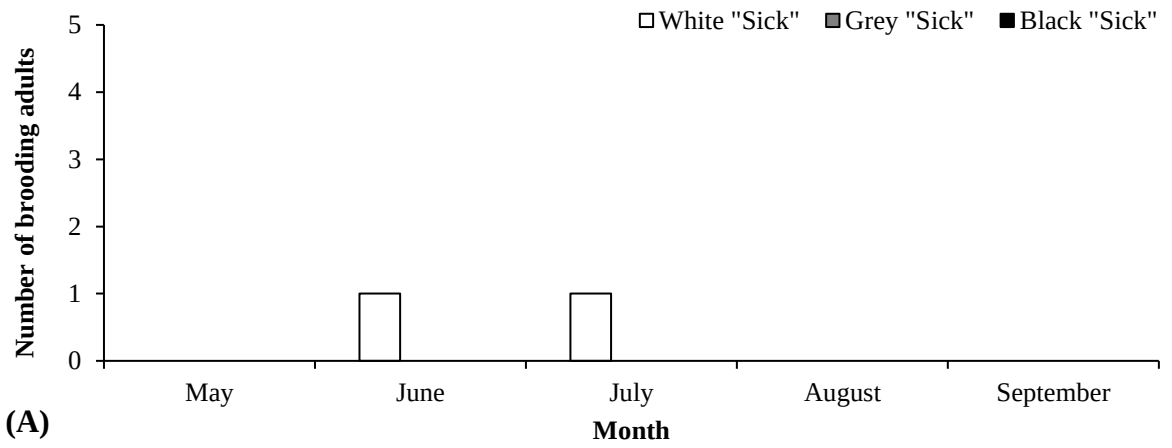
4.3.1.7. Langstone Harbour

Langstone Harbour accounted for the second highest number of brooding adults observed in 2017. Eight individuals were observed, two containing white "sick" in May (6.7

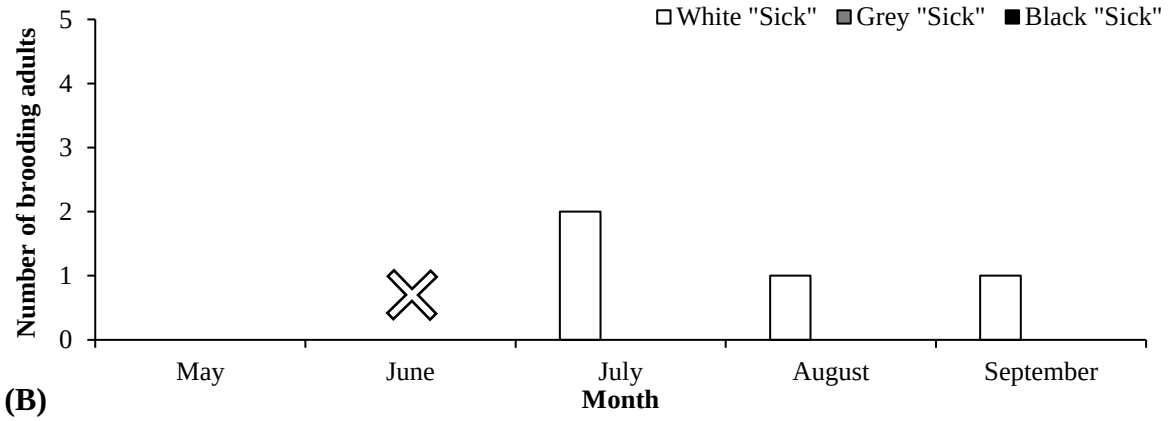
%), three containing white “sick” and two containing grey “sick” in June (16.7 %) and one individual containing grey “sick” in July (3.3 %) (Fig. 4.5E).

4.3.1.8. Sparkes

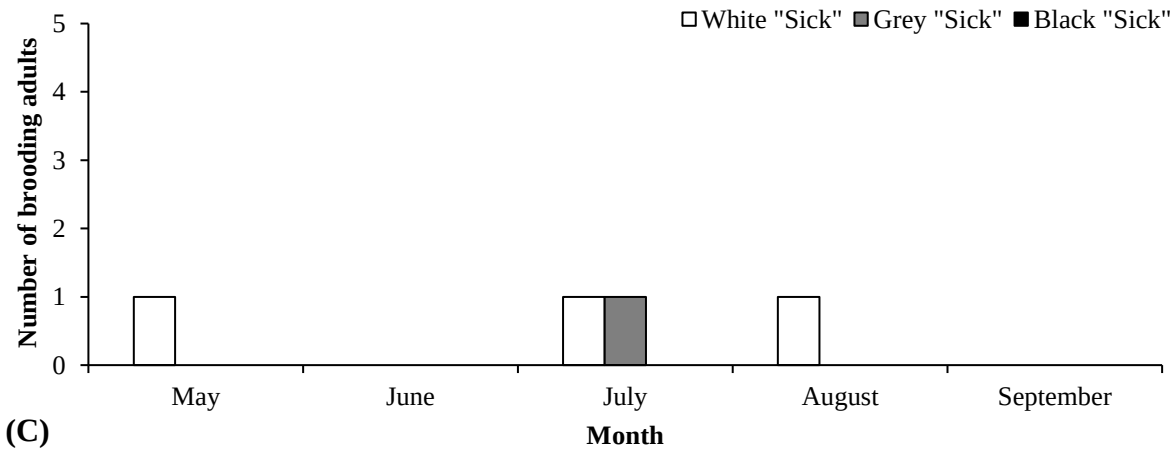
The greatest number of brooding adults throughout 2017 occurred at Sparkes Marina. In total nine individuals were observed, one in May containing white “sick” (3.3 %), the other eight occurred in June (26.7 %) with four white and four grey “sick” broods (Fig. 4.5F).



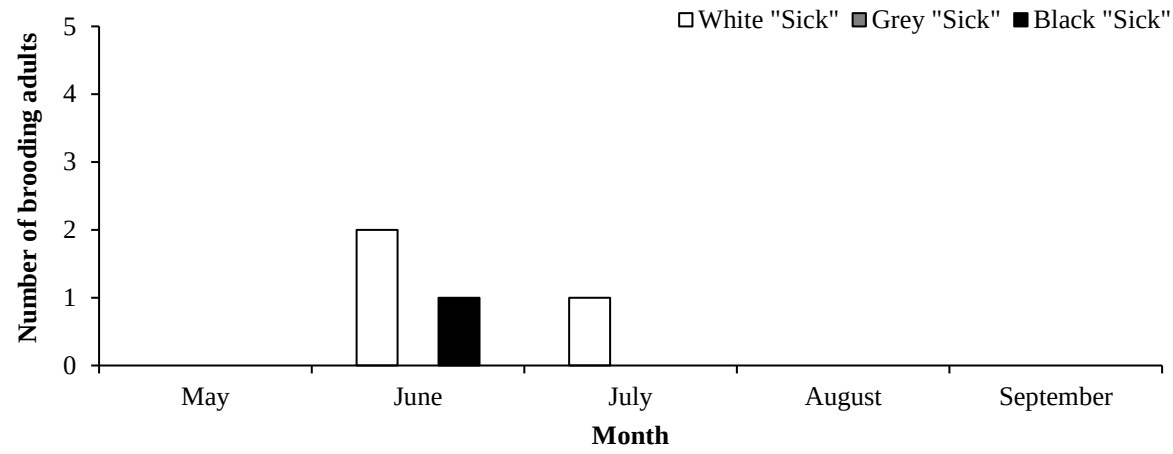
(A)



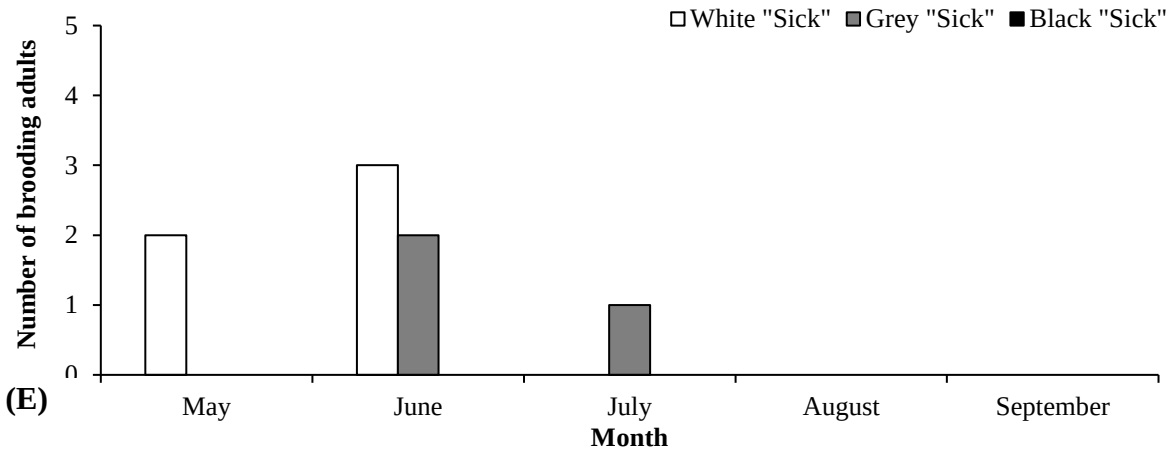
(B)



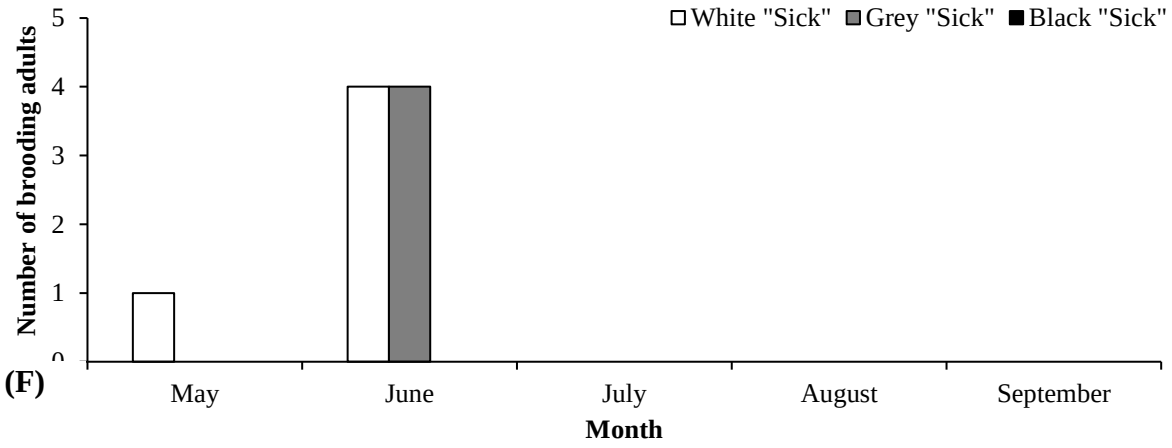
(C)



(D)



(E)



(F)

Figure 4.5. The number of individuals observed brooding the white, grey or black "sick" stages of larval development throughout the 2017 brooding period in (A) Saxon Wharf, (B) Port Hamble Marina, (C) Hamble Point Marina, (D) Portsmouth Harbour, (E) Langstone Harbour and (F) Sparkes Marina.

4.3.1.9. Density comparisons

When pooled from all six locations, 4 ±0.8 % (mean ±SE) of the monitored full-density population were observed brooding larvae (all stages) throughout the 2017 brooding period. Of the monitored half-density population, 4.8 ±1.2 %, were observed brooding larvae (all stages) throughout the 2107 brooding period (Fig. 4.6). No significant difference was observed between the two densities ($p > 0.05$).

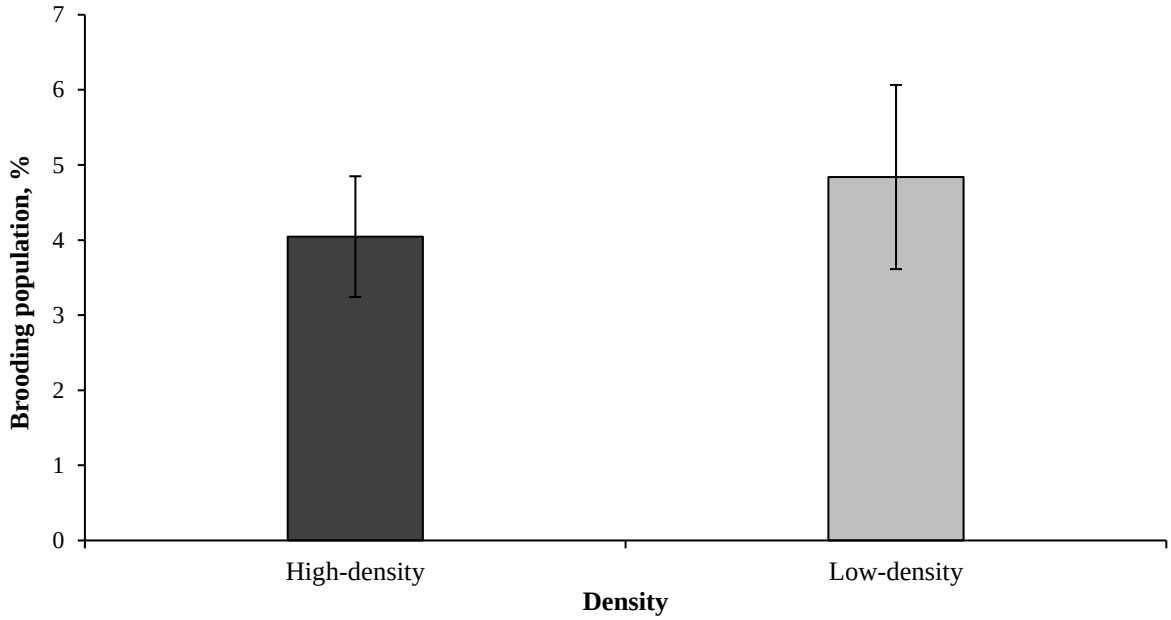
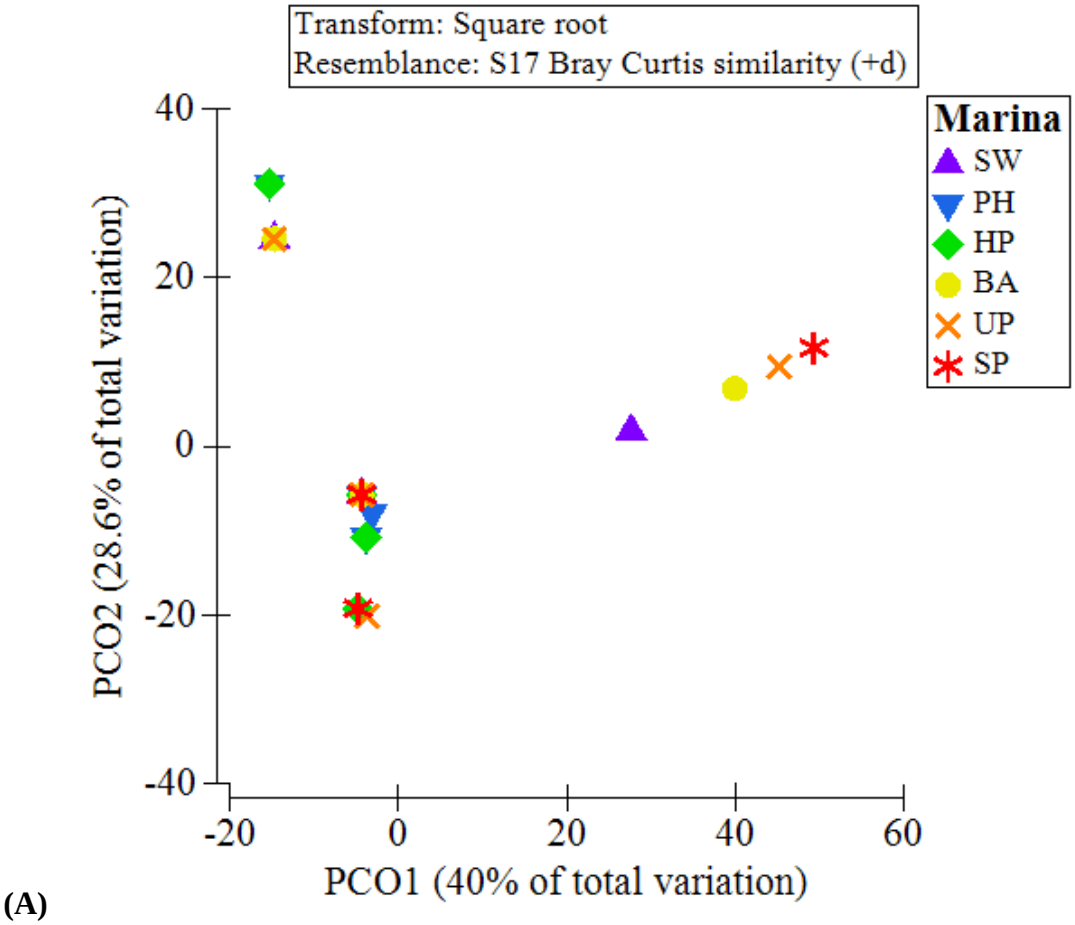


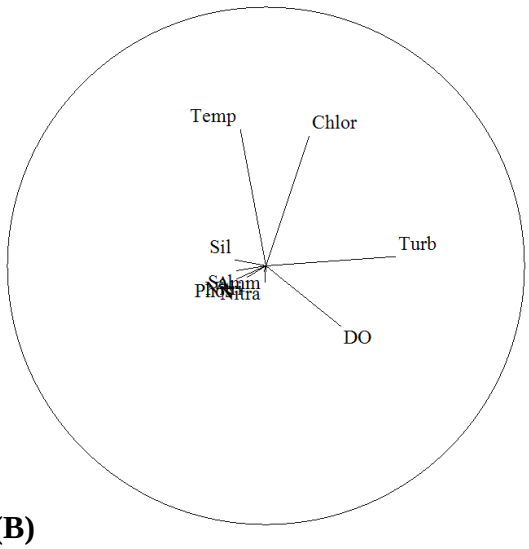
Figure 4.6. Percentage of broodstock populations (mean ±SE) in full- and half-density (n = 354 / density) cages that were observed brooding larvae during the 2017 brooding period. Data pooled from all locations.

4.3.2. Influence of environmental conditions on brooding period

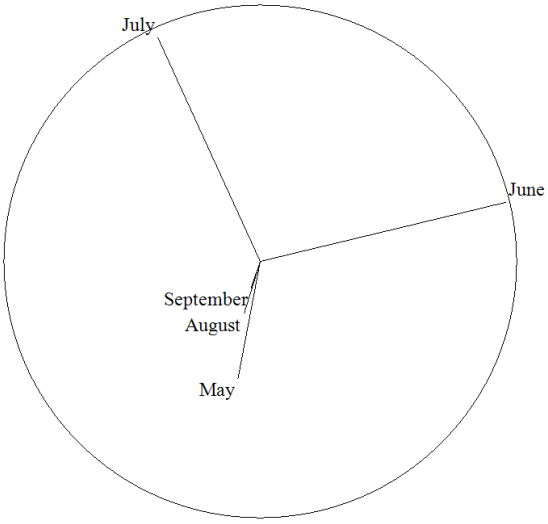
From the environmental parameters available the multivariate analysis indicated that 68.6 % of the data were explained in the two axes. The data formed cluster for each month with the number of brooding adults in June associated with turbidity, July with temperature and chlorophyll and September, August and May associated with nutrients (nitrite, nitrate, silicate and phosphate) and salinity (Fig. 4.7).



(A)



(B)



(C)

Figure 4.7. Principal coordinate analysis (PCO) expressed as ordinations. (A) The occurrence of larval brooding by broodstock oysters in, relation to; (B) available Environment agency water quality data from the most geographically relevant sampling location for each location, and; (C) month during the 2017 spawning season. The strongest relationship explaining the scatter of brooding activity is correlated with temperature for July and turbidity for June. Codes: SW - Saxon Wharf; PH - Port Hamble, HP - Hamble Point; BA - Portsmouth Harbour; UP - Langstone Harbour; SP - Sparkes Marina.

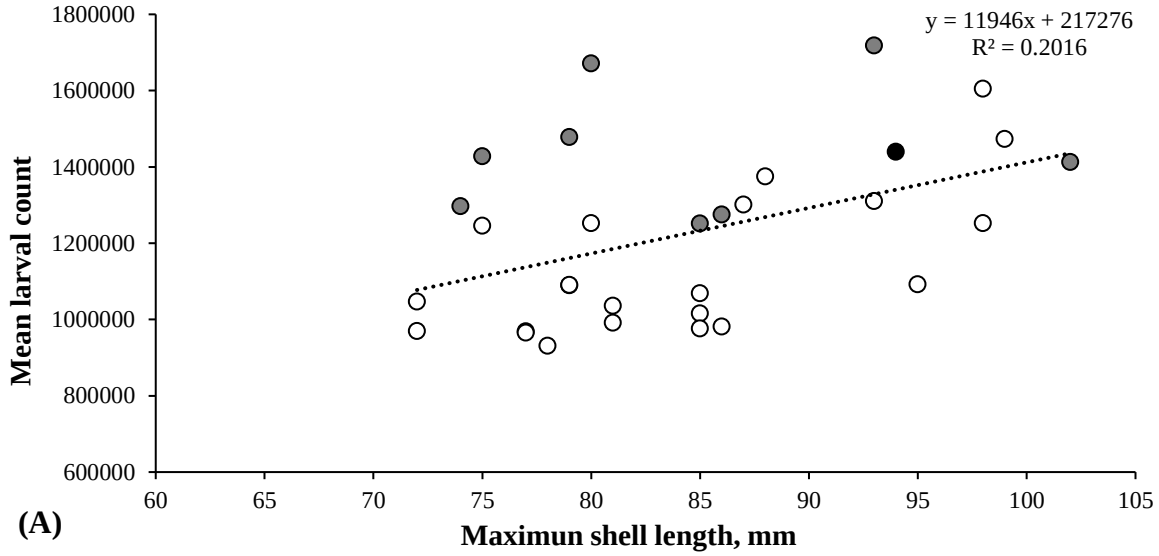
4.3.2. Broodstock fecundity

4.3.2.1. Total population

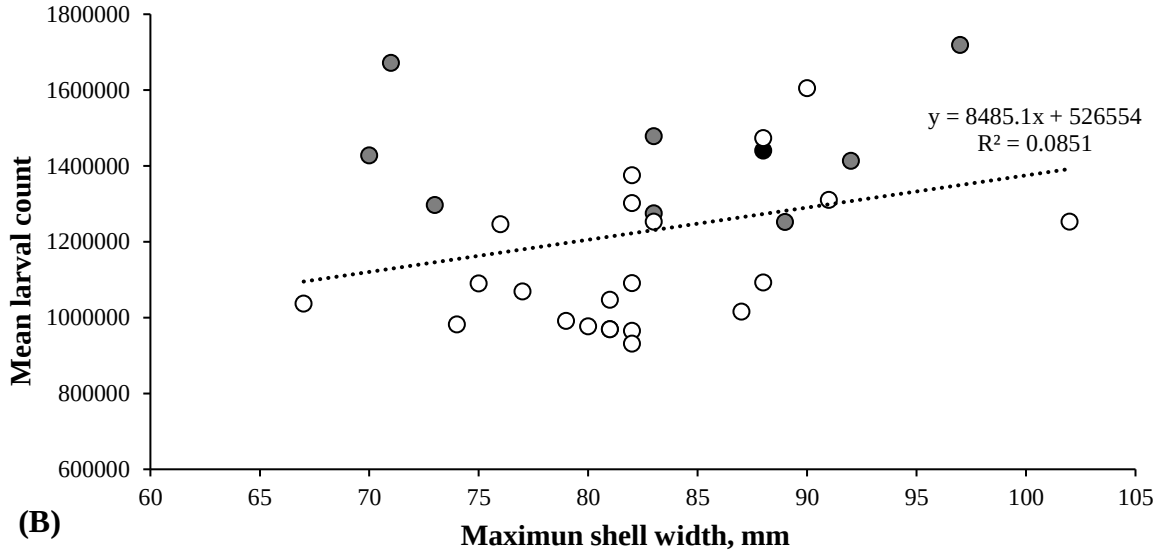
Broodstock fecundity in 2017 ranged from 931,310 ±10,992 - 1,718,536 ±26,844 larvae (mean ±SE) within oysters that ranged from 72 - 102 mm in maximum shell length (mean 84.5 ±1.5), 67 - 102 mm in maximum shell width (mean 82.5 ±1.4), 17 - 40 mm in maximum shell depth (mean 26.9 ±1.0) and 59.1 - 223.9 g in whole wet weight (mean 110.7 ±7.4). Across all locations 22 broods of white sick (71.0 %), eight broods of grey sick (25.8 %) and one brood of black sick (3.2 %) were analysed with a mean larval count of 1,226,168 ±40,580. When estimating total larvae produced from the 958 adults estimated to be brooding in 4.3.1.1, then an approximate estimation can be made that a total of 1,174,668,944 larvae will have been brooded throughout the 2017 brooding period.

Broods of white “sick” occurred within adult oysters measuring 72 - 99 mm, 67 - 102 mm, 17 - 40 mm and 59.1 - 223.9 g in maximum shell length, width, depth and whole wet weight, respectively. Broods of grey “sick” occurred within adult oysters measuring 74 - 102 mm, 70 - 97 mm, 21 - 36 mm and 64.0 - 159.5 g in maximum shell length, width, depth and whole wet weight, respectively. The only black “sick” brood occurred in an adult oyster measuring 94 mm, 88 mm, 29 mm and 125.6 g in maximum length, width, depth and whole wet weight, respectively.

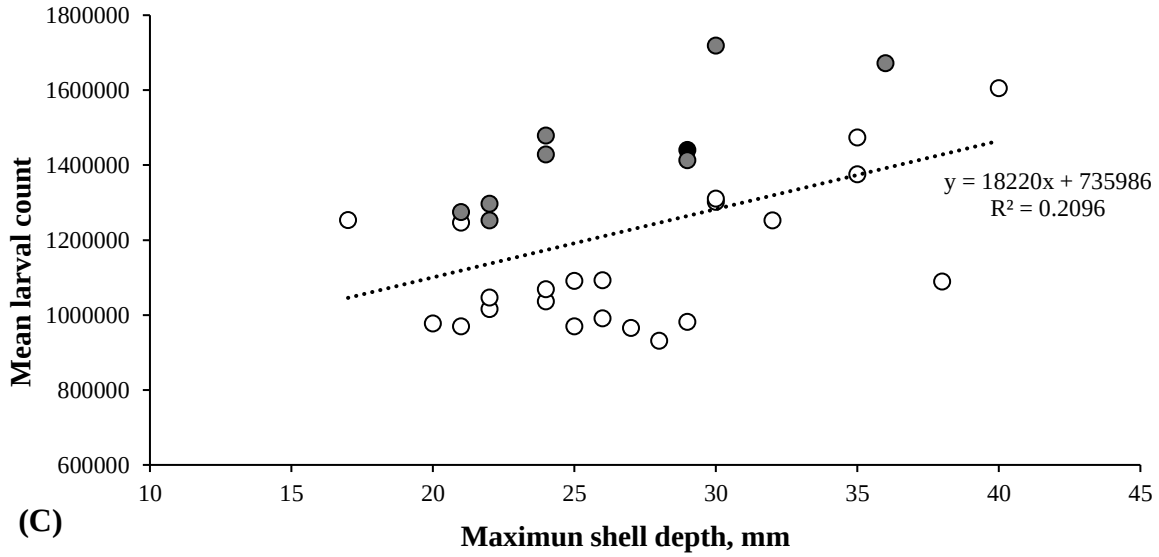
There was a positive correlation between larval brood size and all independent parameters, which was significant for length (Pearson correlation, $r = 0.449$, n = 31, $p = 0.011$), depth ($r = 0.459$, n = 31, $p = 0.010$) and weight ($r = 0.628$, n = 31, $p < 0.001$). There was no correlation with larval brood size and width ($r = 0.292$, n = 31, $p = 0.111$) (Fig. 4.8).


y = 11946x + 217276
R² = 0.2016
Mean larval count
1800000
1600000
1400000
1200000
1000000
800000
600000
60
65
70
75
80
85
90
95
100
105
Maximun shell length, mm

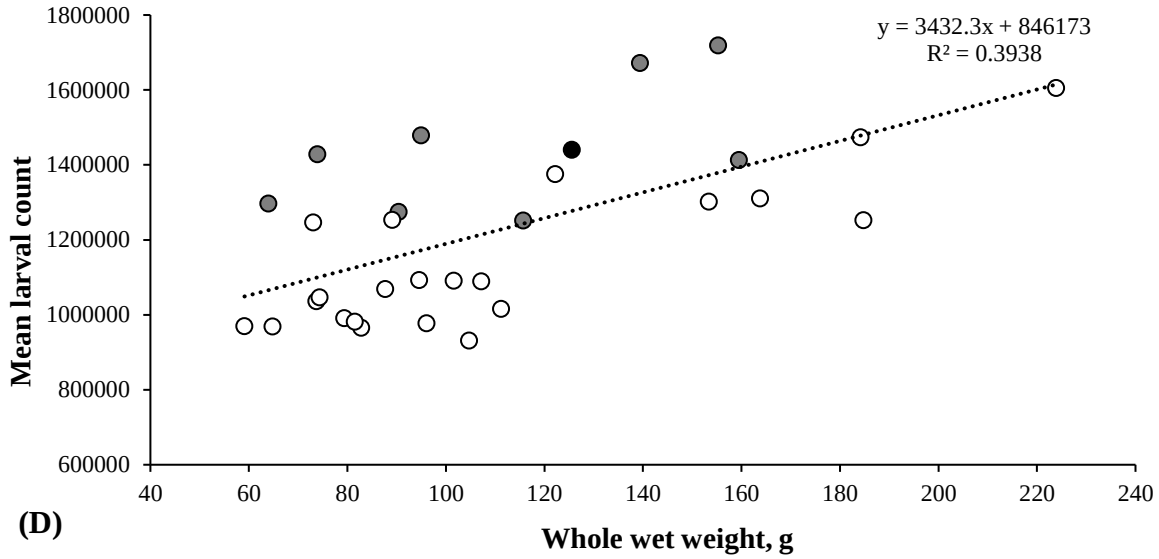
(A)


y = 8485.1x + 526554
R² = 0.0851
Mean larval count
1800000
1600000
1400000
1200000
1000000
800000
600000
60
65
70
75
80
85
90
95
100
105
Maximun shell width, mm

(B)


y = 18220x + 735986
R² = 0.2096
Mean larval count
1800000
1600000
1400000
1200000
1000000
800000
600000
10
15
20
25
30
35
40
45
Maximun shell depth, mm

(C)



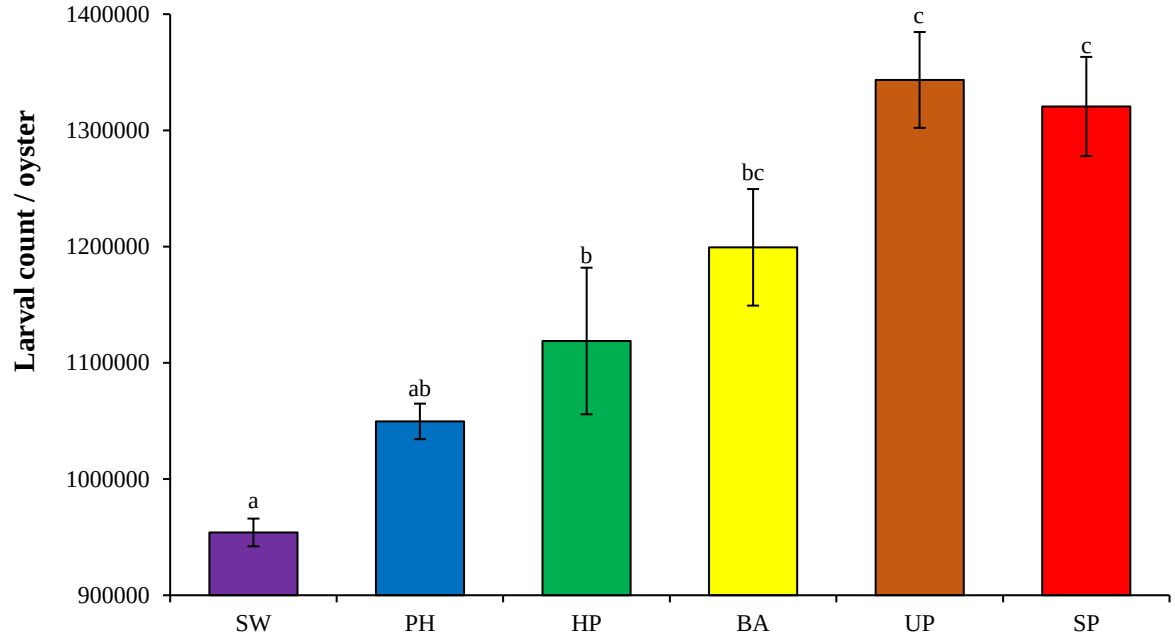
(D)

Figure 4.8. The relationship between mean larval counts per brooding adults and (A) maximum shell length, (B) maximum shell width, (C) maximum shell depth and (D) whole wet weight of the respective adults. The colouration of each data point indicates larval stage - white, grey or black "sick". Data pooled from all locations in 2017 (n = 31).

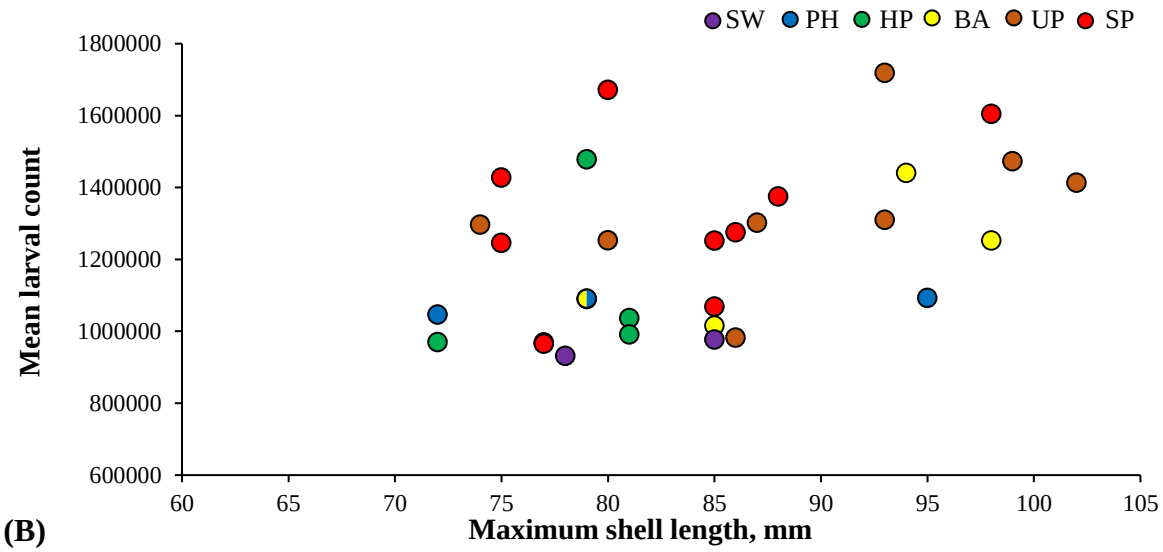
4.3.2.3. Comparison between locations

A general trend of increasing brood size was observed from west to east with regards to geographical location. The adults in the area closest to the source location (UP) were found to be brooding the most larvae 1,343,394 ±41,233 (mean ±SE), followed by those at SP (1,320,569 ±42,655), BA (1,199,350 ±50,147), HP (1,118,772 ±63,083), PH (1,049,600 ±15,283) and SW that produced the fewest larvae per adult (954,027 ±11,934) (Fig. 4.9A).

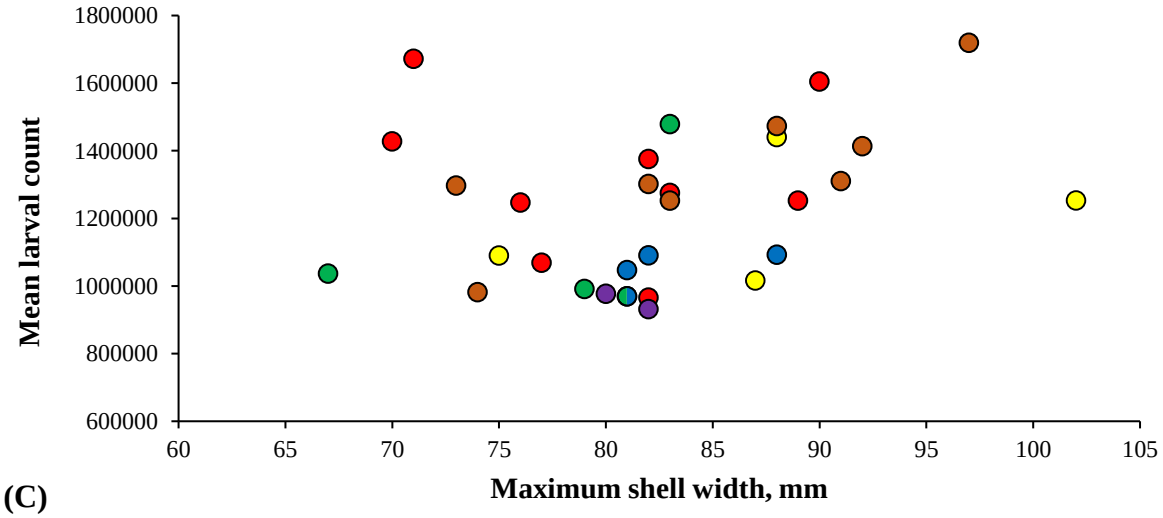
The largest brood across all locations contained 1,718,536 ±26,844 larvae (mean ±SE) was found within an oyster from the UP population measuring 93 mm, 97 mm, 30 mm and 155.3 g in maximum shell length, width depth and whole wet weight, respectively. The smallest brood contained 931,310 ±10,992 larvae and was within an oyster from the SW population that measured 78 mm, 82 m, 28 mm and 104.7 g in maximum shell length, width depth and whole wet weight, respectively (Fig. 4.9B-E).


Larval count / oyster
1400000
1300000
1200000
1100000
1000000
900000
a
ab
b
bc
c
c
SW
PH
HP
BA
UP
SP
Marina location

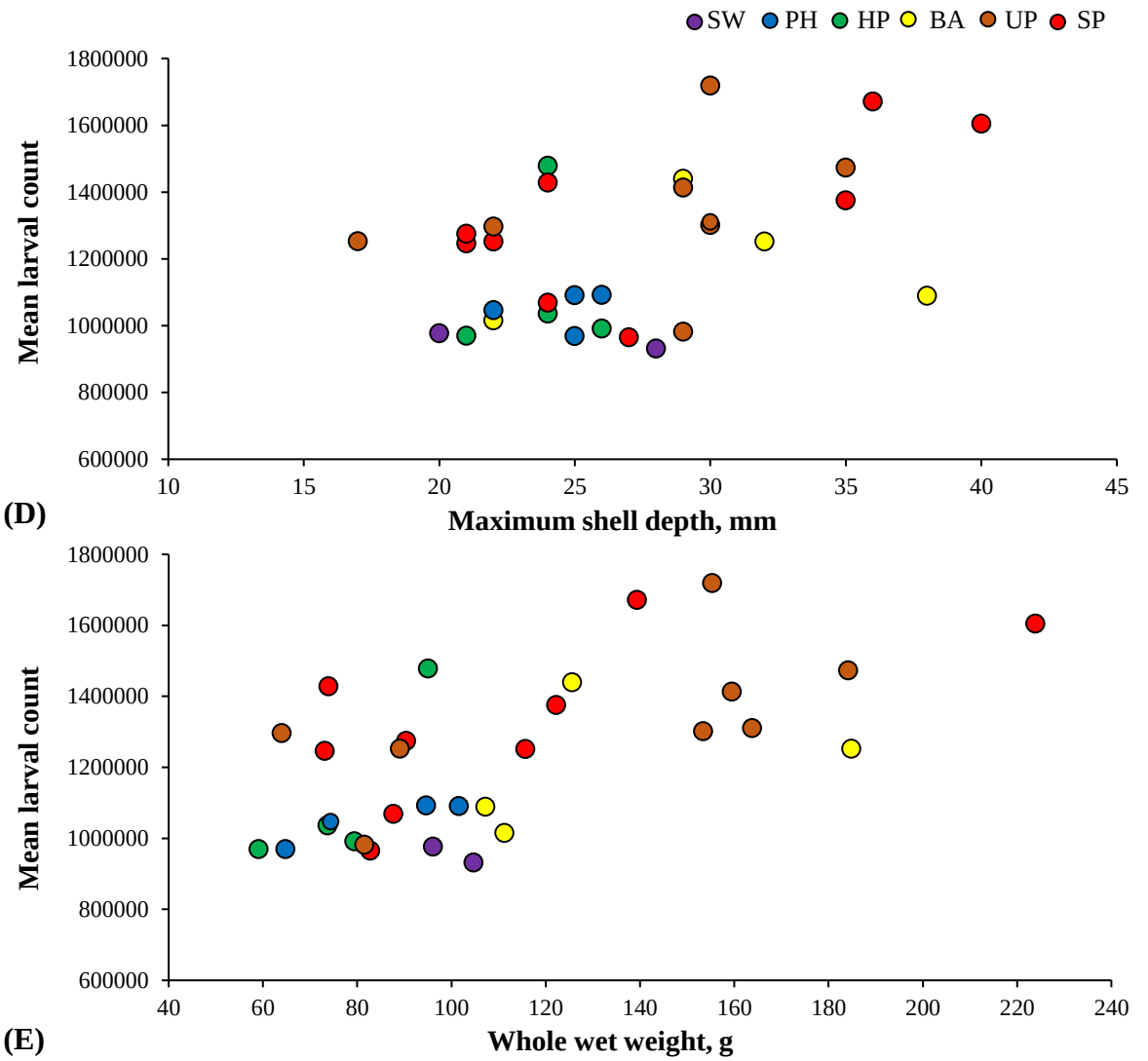
(A)


SW
PH
HP
BA
UP
SP
Mean larval count
1800000
1600000
1400000
1200000
1000000
800000
600000
60
65
70
75
80
85
90
95
100
105
Maximum shell length, mm

(B)


Mean larval count
1800000
1600000
1400000
1200000
1000000
800000
600000
60
65
70
75
80
85
90
95
100
105
Maximum shell width, mm

(C)



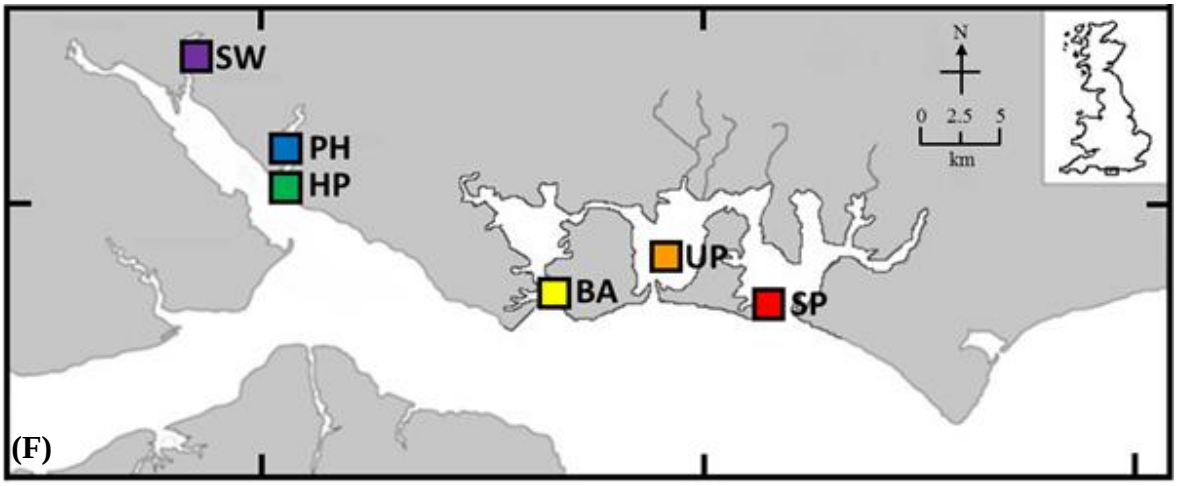


Figure 4.9. (A) Broodstock larval counts / oyster (mean ±SE) compared across all six sampling locations. Mean larval counts per oyster in relation to (B) maximum shell length, (C) maximum shell width, (D) maximum shell depth and (E) whole wet weight of brooding adults from (F) the six sample locations across the Solent with colours relating to marina location. Source population of broodstock was obtained from the commercial fishery near UP. Codes: SW - Saxon Wharf (n = 2), PH - Port Hamble (n = 4), HP - Hamble Point (n = 4), BA - Portsmouth Harbour (n = 4), UP - Langstone Harbour (n = 8), SP - Sparkes Marina (n = 9).

4.3.2.4. Monthly comparisons

Larval brood size began at 1,141,402 ±78,071 (mean ±SE) in May and increased to its peak in June of 1,324,673 ±55,361. After this peak the larval count decreased into July and August to 1,113,988 ±75,118 and 1,018,805 ±27,688, respectively (Fig. 4.10). Month was determined to signigicantly impact the quantity of larvae brooded (GLM, $p < 0.001$).

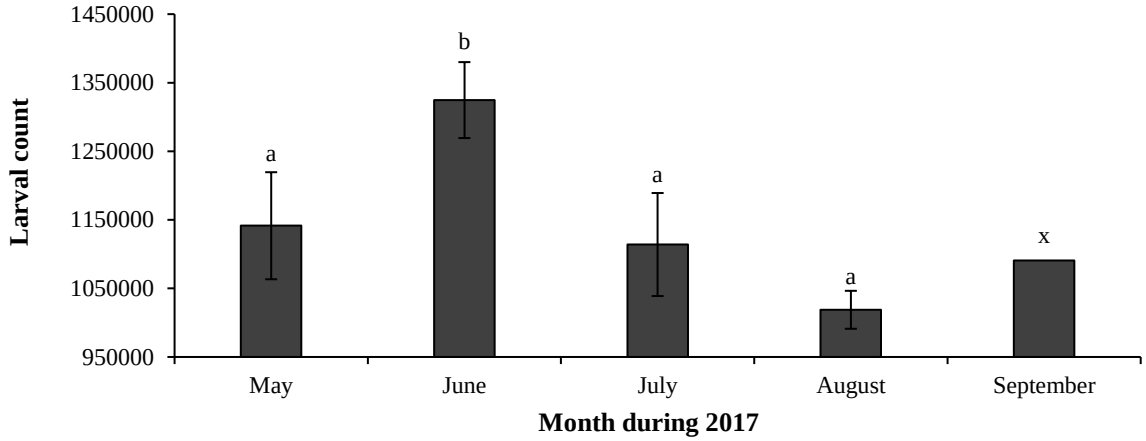


Figure 4.10. Comparative larval counts (mean ±SE) between brooding adult populations for May (n = 4), June (n = 17), July (n = 7), August (n = 2) and September (n = 1) 2017, pooled from all locations. Lower-case data labels indicate significant differences between the populations ($p < 0.05$), x indicates analysis was not available.

4.3.2.5. Density comparisons

Full-density populations contained 1,121,175 ±24,268 larvae (mean ±SE), significantly fewer larvae than those from half-density populations pooled from all locations, 1,312,633 ±33,051 (GLM, b +/- SE = 0.0003964, $Z = 363.38$, $p < 0.001$) (Fig. 4.11).

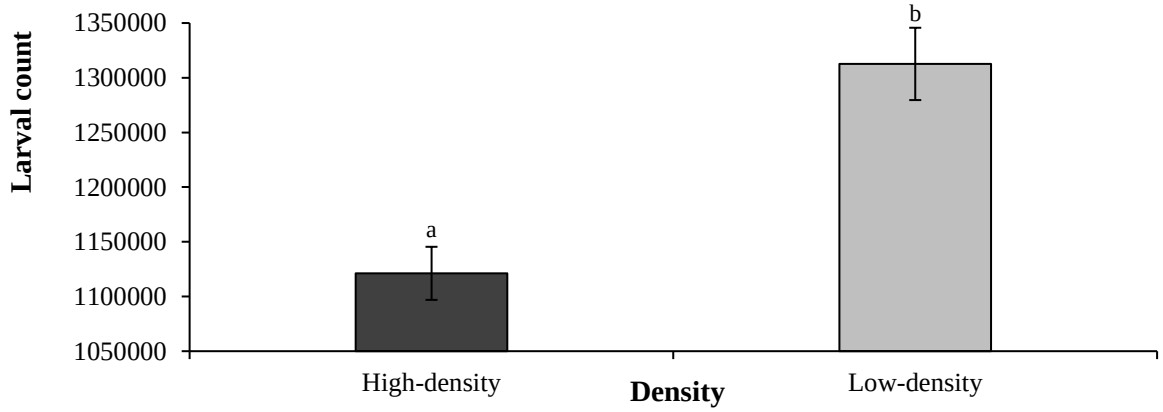


Figure 4.11. Larval counts (mean ±SE) from brooding oysters in full- (n = 14) and half-density (n = 17) populations during the 2017 brooding period, data pooled from all locations. Lower-case data labels indicate significant differences between populations ($p < 0.05$).

4.3.3. Influence of environmental conditions on larval brood size

From the environmental parameters available the multivariate analysis indicated that 65.7 % of the data were explained in the two axes. The data formed cluster for each month with the quantity of larvae brooded in June associated with turbidity and July with temperature, the other months were shown not to be strongly associated with any of the environmental parameters (Fig. 4.12).

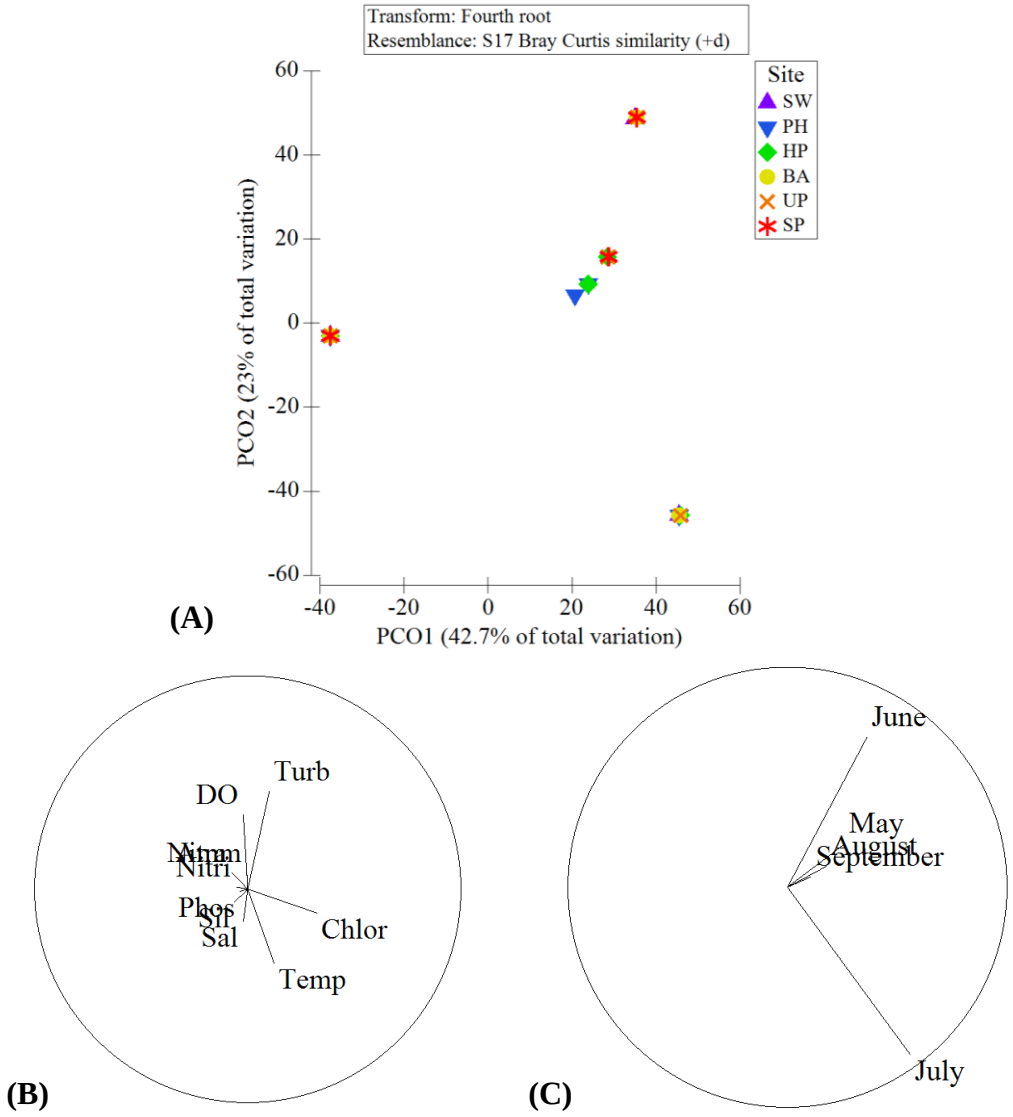


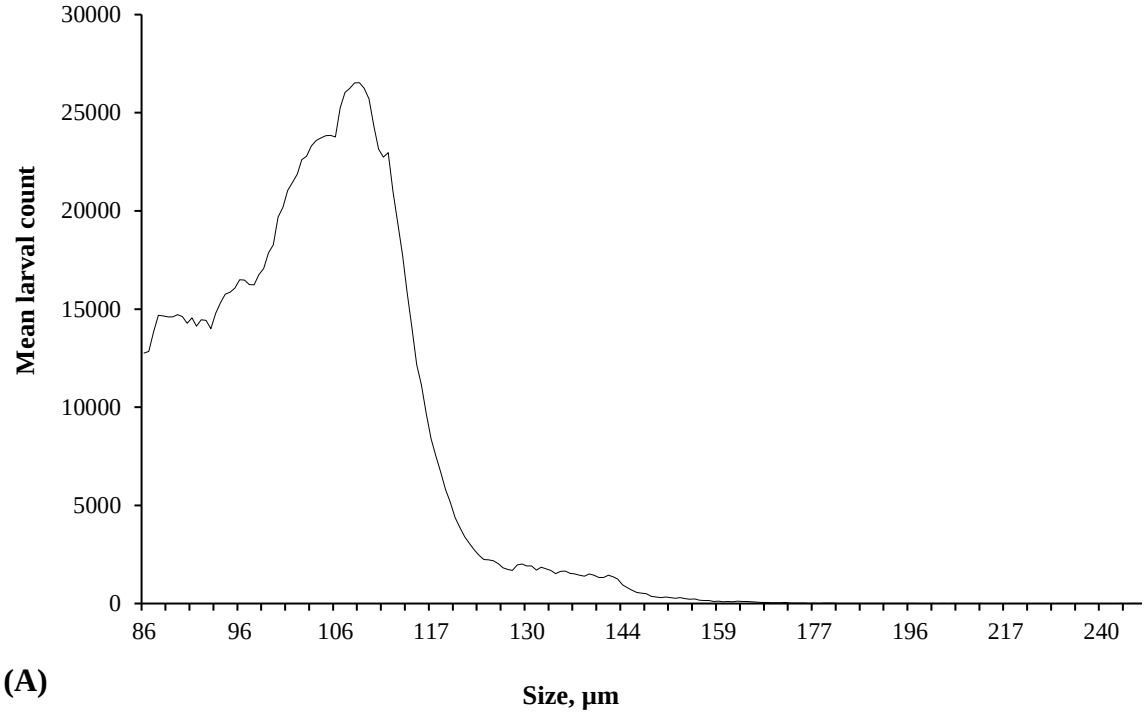
Figure 4.12. Principal coordinate analysis (PCO) expressed as ordinations. (A) The quantity of larvae brooded by adult oysters in, relation to; (B) available Environment agency water quality data from the most geographically relevant sampling location for each location, and; (C) month during the 2017 spawning season. The strongest relationship explaining the scatter of brooding activity is correlated with temperature for July and turbidity for June. Codes: SW - Saxon Wharf; PH - Port Hamble, HP - Hamble Point; BA - Portsmouth Harbour; UP - Langstone Harbour; SP - Sparkes Marina.

4.3.4. Larvae size comparisons

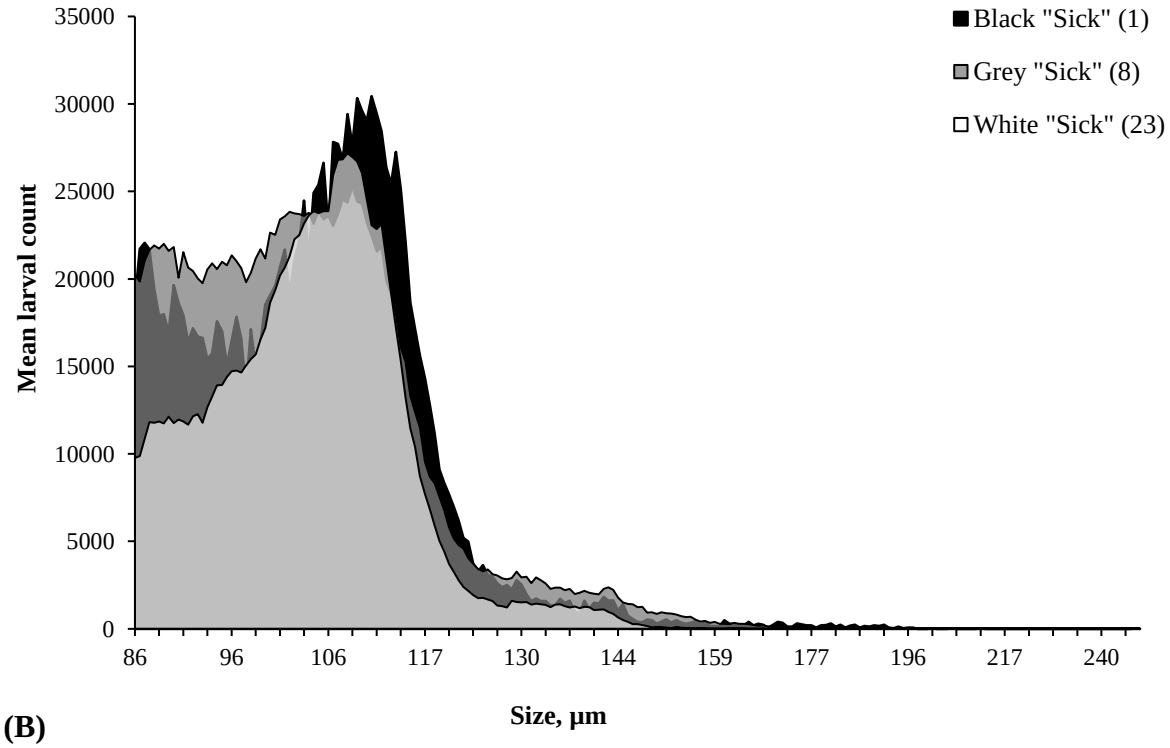
4.3.4.1. Total population

The particle sizes of the larvae, produced by the Multisizer™ 3, were smaller than the reported sizes for the larvae stages measured. This reduction in size was due to the dehydration that occurred during the preservation process in ethanol. As all samples underwent the same treatment, the reduction in size will have occurred within all larvae analysed but may not have occurred in the same manner within different stages of larval development. Thus, determination of differences in size of larvae was compared relative to other samples, without reference to reported larval sizes.

Larval sizes across all locations and developmental stages ranged from the minimum set threshold of 86 µm, to 249 µm. The most frequent size grouping for all larvae from all locations was 108 - 108.5 µm (26,531 individuals, mean) (Fig 4.13A). The most frequent size for larval broods of white "sick" stage larvae was also 108 - 108.5 µm, the most frequent size for larval broods of grey "sick" stage larvae was 108.5 - 109 µm (25,224 individuals) and for the brood of black sick was 111 - 111.5 µm (30,443 individuals) (Fig. 4.13B). The larvae within white "sick" broods were more evenly distributed with the size range compared with the grey and black "sick" broods.



(A)



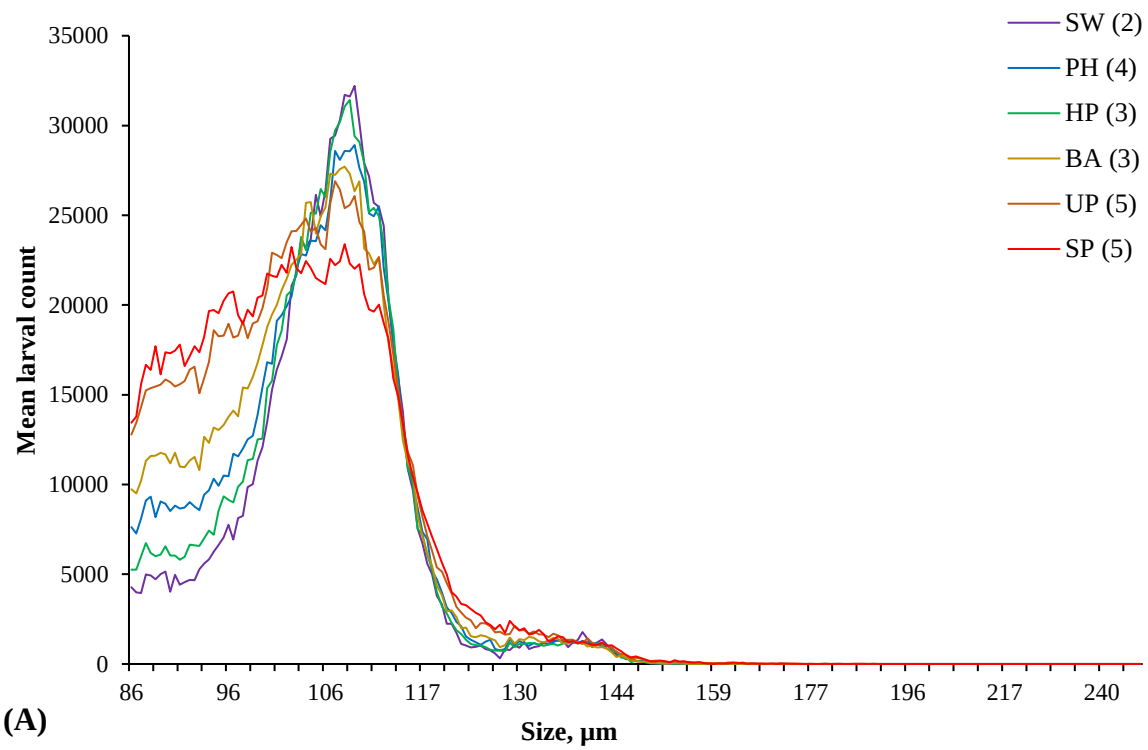
(B)

Figure 4.13. Larval size groupings of (A) all larval developmental stages from all brooding adults at all six sampling locations (n = 31) and (B) the variation between white (n = 22), grey (n = 8) and black (n = 1) "sick" larval stages from all brooding adults at all six sampling locations.

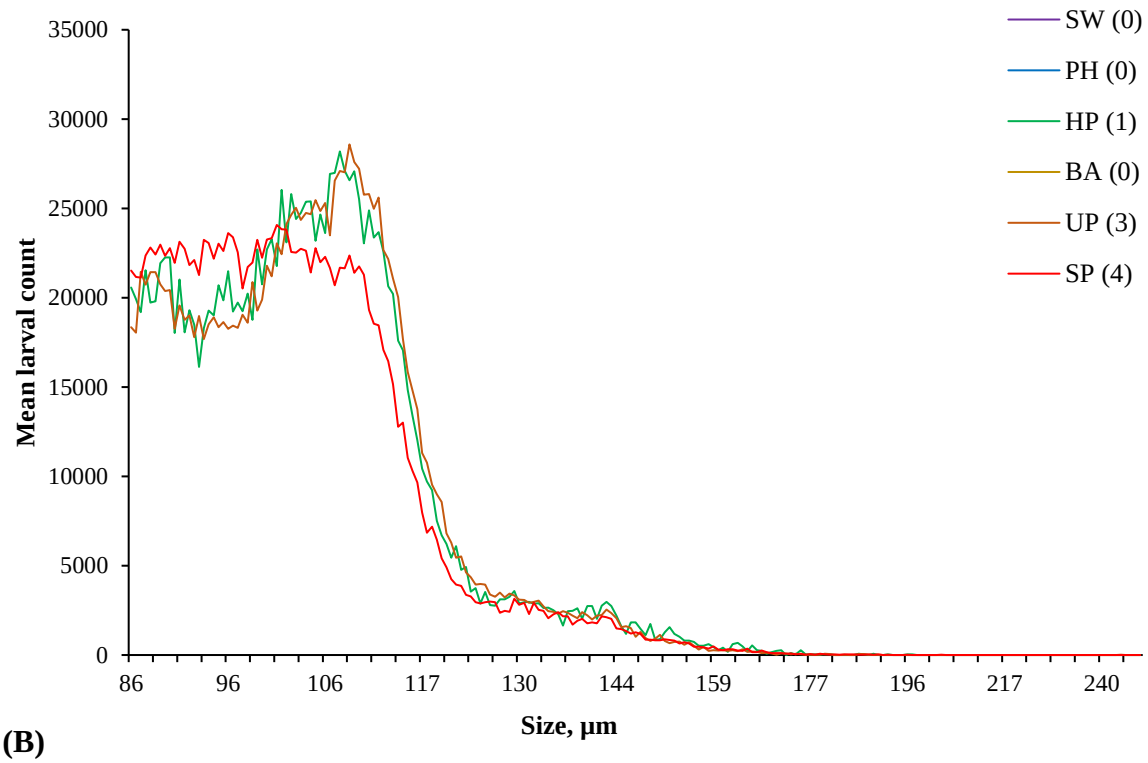
4.3.4.3. Individual location comparisons

The size distribution of white “sick” larvae from broodstock located at SW, PH, HP and BA was relatively limited with each 0.5 µm size grouping from 100 - 114, 99 - 114, 99.5 - 114 and 97 - 113.5 µm, respectively, comprising of > 15,000 larvae. In comparison, larval size groupings occurring between the size range of 87.5 - 114 and 87 - 113.5 µm comprised of > 15,000 larvae per 0.5 µm grouping within broodstock from UP and SP, respectively (Fig. 4.14A).

The size distribution of grey “sick” larvae was only available from broodstock located at HP, UP and SP. The general demographic of the larvae from the three populations more closely resembled each other than they did for the white “sick” larval broods, with larval size groupings occurring between the size range of 86 - 115, 86 - 115.5 and 86 - 113.5 µm comprising of > 15,000 larvae per 0.5 µm grouping at HP, UP and SP, respectively (Fig. 4.14B). No comparison was available for black “sick” larval stage due to the presence of one sample from an adult in the BA population.



(A)



(B)

Figure 4.14. Comparison of larval size groupings of (A) white "sick" and (B) grey "sick" from individual marina locations. Codes: SW - Saxon Wharf, PH - Port Hamble, HP - Hamble Point, BA - Portsmouth Harbour, UP - Langstone Harbour, SP - Sparkes Marina. Sample n shown in parentheses.

4.3.4.4. Density comparisons

The size distribution of all larvae (white and grey "sick") from full-density broodstock was relatively limited with each 0.5 µm size grouping from 98 - 113.5 µm comprising of 15,000 - 28,000 larvae. In comparison, half-density broodstock larval size groupings occurring between the size range of 86 - 114.5 µm all comprised of > 15,000 larvae per 0.5 µm grouping (Fig. 4.15A).

A similar trend is observed when comparing the white "sick" larvae alone with each 0.5 µm grouping between 98.5 - 114.0 µm comprised of > 15,000 larvae for the full-density populations. The same quantity of larvae was found in each 0.5 µm size grouping between 93.5 - 113.0 µm from the half-density populations (Fig. 4.15B).

The demographic of the grey "sick" larvae from the full- and half-density populations more closely resembled one another, with each 0.5 µm grouping between 86 - 112.0 µm comprising of > 15,000 larvae for the full-density populations. The 0.5 µm grouping between 86 - 115.5 µm comprised of > 15,000 larvae for the half-density populations (Fig. 4.15C). No comparison was available for black "sick" larval stage due to the presence of one sample from an adult in one of the half-density populations.

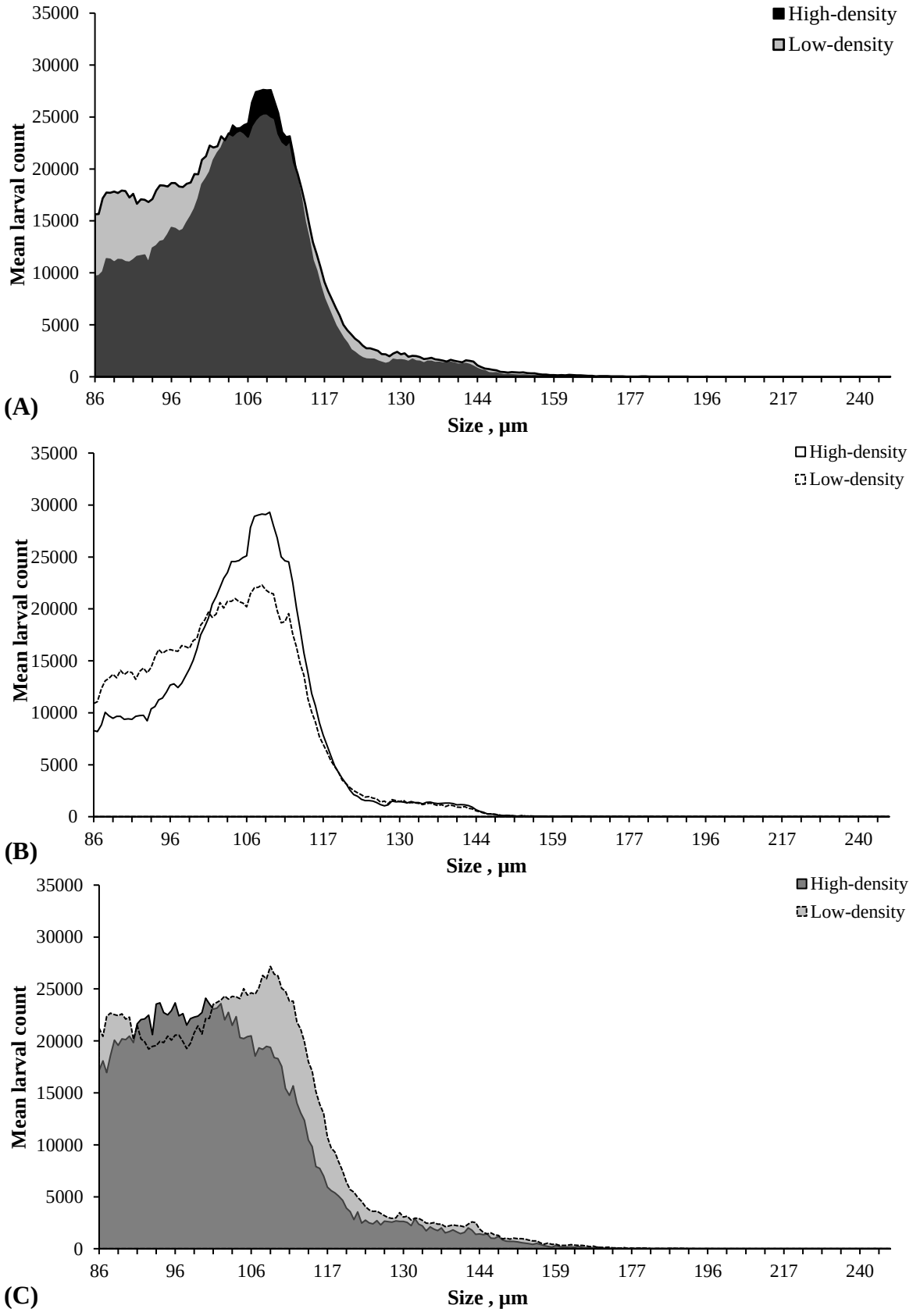


Figure 4.15. Comparison of larval size groupings of (A) combined larval developmental stages, full-density n = 13, half-density n = 18, (B) white "sick" developmental stage, full-density n = 11, half-density n = 11 and (C) grey "sick" developmental stage, full-density n = 2, half-density n = 6, from brooding adults.

4.3.5. Larval settlement

4.3.5.1. 2017 Settlement

A total of six spat were observed adhered to settlement plates that were initially deployed in July 2017. Of these six spat, two were morphologically and genetically identified as *O. edulis* (Fig. 4.16) and four were morphologically and genetically identified as *C. gigas*. Both *O. edulis* spat were present on settlement plates at GP, two of the *C. gigas* were located at SW and two at SP. No settlement of either oyster species was observed at HY, OV, MC, SS, NT, PH, HP, BA or UP (Fig. 4.23A). Settlement of *O. edulis* was 0.2 ±0.1 spat / plate (mean ±SE) at GP. Settlement of *C. gigas* per plate was also 0.2 ±0.1 at both SW and SP (Fig. 4.23B). The two *O. edulis* spat were both found on horizontally orientated settlement plates and provided a mean of 0.03 ±0.02 spat / plate. The four *C. gigas* spat were also all found on horizontally orientated settlement plates and provided a mean of 0.06 ±0.03 spat / plate (Fig. 4.23C). Only one individual *C. gigas* was found on the upper surface of the settlement plates with the other five spat all attaching to the under surface of the settlement plates. No significant differences were observed between orientations, species or orientation and species (Kruskal-Wallis H test, H (3) = 7.463, p = 0.59) or location ($p > 0.05$).

Figure 4.16. *Ostrea edulis* spat settled to the underside (smooth) of a horizontally deployed settlement plate. Image captured using a Leica EZ4W stereo microscope.

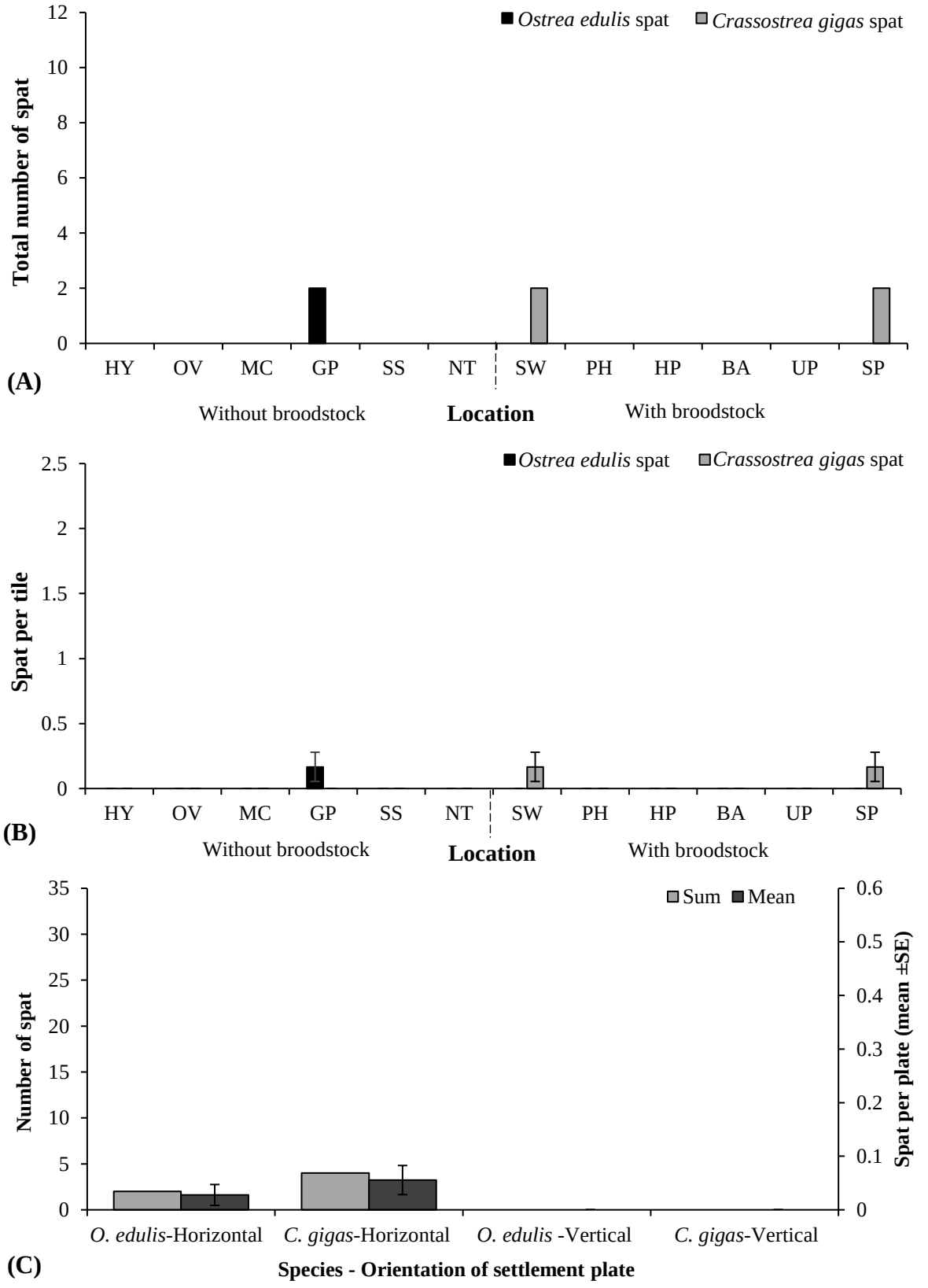
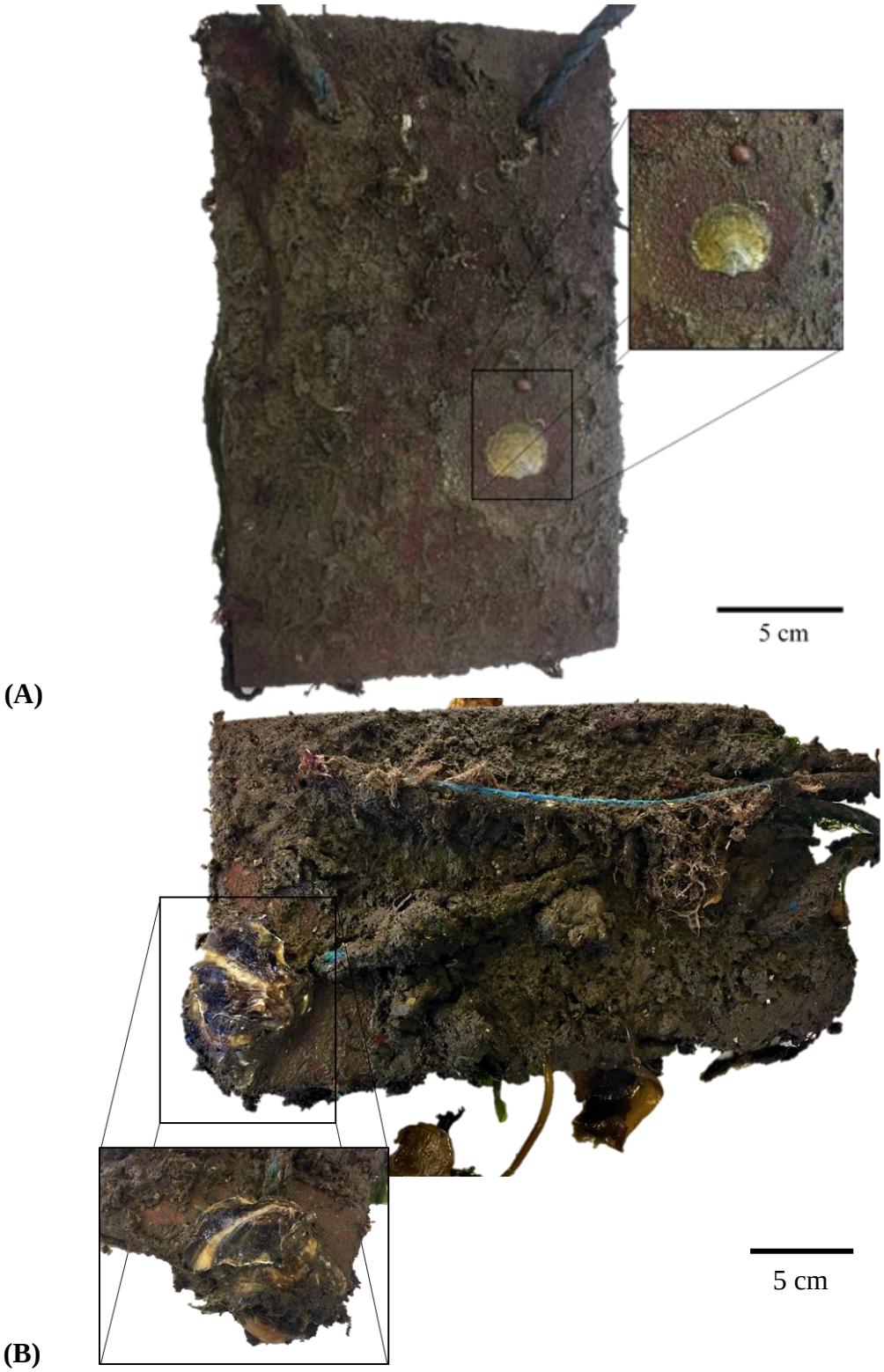


Figure 4.16. (A) Total settlement and (B) individual plate settlement (mean ±SE), of *Ostrea edulis* and *Crassostrea gigas* spat on settlement plates deployed in marina locations across the Solent, with and without broodstock cages suspended at the locations in 2017. (C) Settlement of *O. edulis* and *C. gigas* (mean ±SE) on horizontal and vertical settlement plates at all locations in 2017. No significant differences were observed between settlement at any location where spat were present or between orientations and species ($p < 0.05$).

4.3.5.2. 2018 Settlement

A total of 35 spat were observed adhered to settlement plates that were initially deployed in July 2018. Of the spat observed, two were morphologically identified as *O. edulis* (Fig. 4.17A) and 33 as *C. gigas* (Fig. 4.17B). Of these, eight *C. gigas* were observed at both HY and SW, two *C. gigas* at PH, two *O. edulis* and 11 *C. gigas* at UP, and three *C. gigas* at SP (Fig. 4.18A). No settlement of either oyster species was observed at MC, GP, SS, HP or BA. Settlement plates at OV and NT could not be analysed due to their removal. Settlement of *C. gigas* was 1.3 ±0.8 spat / plate (mean ±SE) at HY, 0.7 ±0.3 at SW, 0.2 ±0.2 at PH, 1.0 ±0.3 at UP and 0.3 ±0.1 at SP. Settlement of *O. edulis* was 0.2 ±0.1 spat / plate at UP. Significantly more *C. gigas* spat were observed settled at HY, SW and UP that at PH and SP ($p < 0.05$), significantly more *C. gigas* spat than *O. edulis* spat were also observed settled at UP ($p < 0.01$) (Fig. 4.18B). The *O. edulis* spat were both found on both a horizontally (n = 1) and a vertically oriented (n = 1) settlement plates and provided a mean of 0.02 ±0.01 spat / plate. Of the 33 *C. gigas*, 27 were found on horizontal settlement plates and provided a mean of 0.41 ±0.12 spat / plate, the other six were found on vertical settlement plates and provided a mean of 0.10 ±0.04 spat / plate (Fig. 4.18C). One *O. edulis* spat was found attached to the upper surface of a settlement plate whilst the other was found attached to the under surface of a settlement plate. Of the 27 *C. gigas* spat on horizontal settlement plates, 13 were found on upper and 14 on under surfaces. Settlement on the rough and smooth surfaces of the vertical settlement plates was evenly distributed with three *C. gigas* spat on each surface. The number of *C. gigas* spat on horizontal plates was significantly greater than the number of *C. gigas* spat on vertical, *O. edulis* spat on horizontal and vertical plates, the number of *C. gigas* spat on vertical plates was also significantly greater than *O. edulis* spat on horizontal and vertical plates (Kruskal-Wallis H test, $p < 0.05$). No significant difference was observed between *O. edulis* spat on horizontal and vertical plates ($p > 0.05$).



(A)

(B)

Figure 4.17. (A) *Ostrea edulis* spat on the rough side of a vertically suspended settlement plate. (B) *Crassostrea gigas* spat on the top side (rough) of a horizontally suspended settlement plate. Tile dimensions: 27 x 16.5 x 1 cm. The spat and surrounding area was manually cleaned to allow for ease of viewing in this figure.

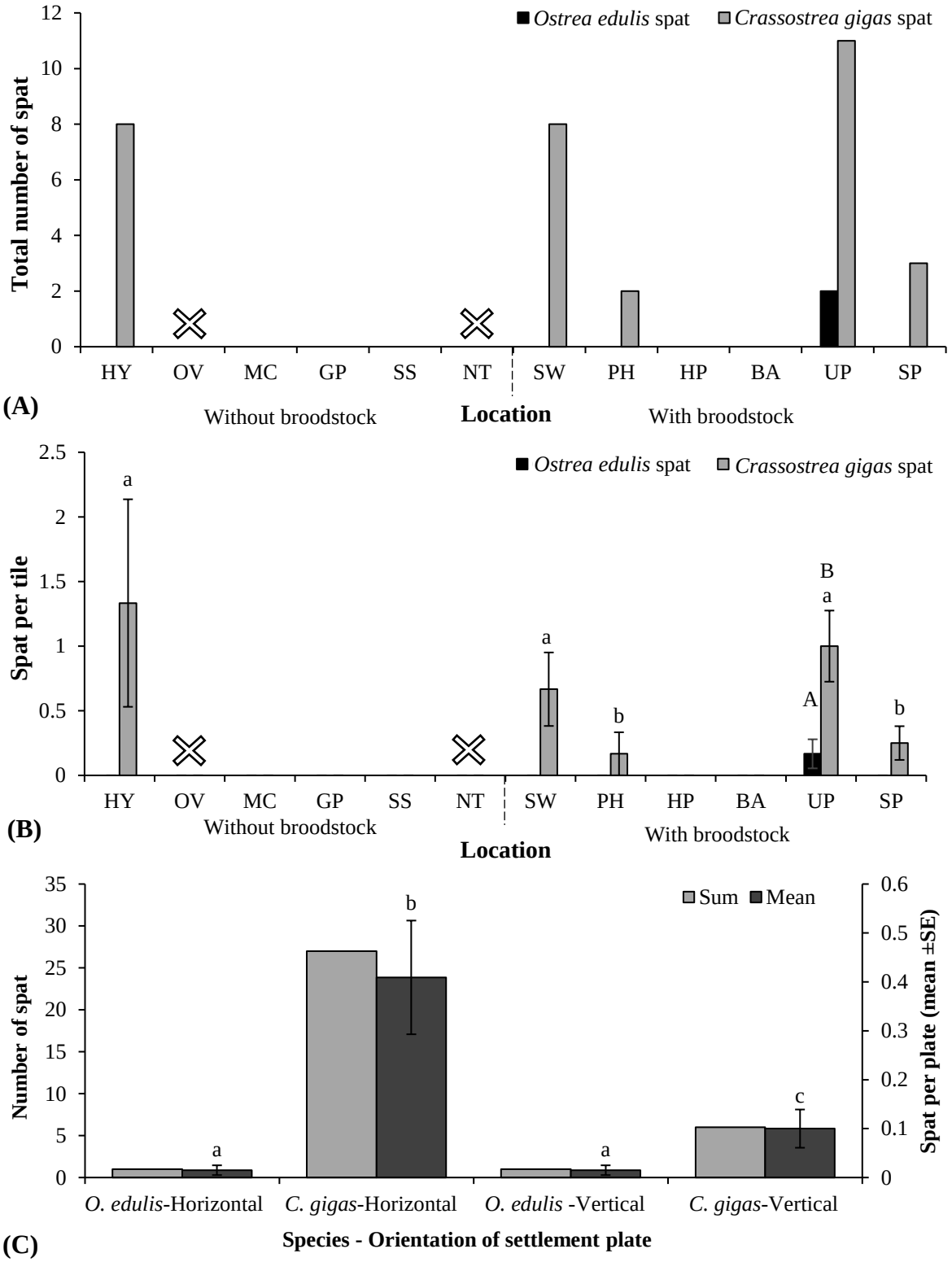


Figure 4.18. (A) Total settlement and (B) individual plate settlement (mean ±SE), of *Ostrea edulis* and *Crassostrea gigas* spat in marina locations across the Solent, with and without broodstock cages in 2018. Locations marked with 'X' indicate loss of settlement plates. Upper-case data labels indicate significant differences between species at the same location and lower-case data labels indicate significant differences between the same species at different locations. (C) Settlement of *O. edulis* and *C. gigas* on horizontal and vertical settlement plates at all locations in 2018. Lower-case data labels indicate significant differences both orientations and species ($p < 0.05$).

No significant differences were observed for settlement of *O. edulis* spat on the same settlement plate orientation between 2017 and 2018 ($p > 0.05$), however, the settlement of *C. gigas* spat observed on horizontal and vertical was significantly greater in 2018 ($H = 4.243$, $p < 0.001$ and $H = 2.539$, $p = 0.011$, respectively).

Large quantities of non-target fouling species were observed adhered onto, or living amongst other species on the settlement plates and the communities formed comprised of numerous species within various phyla including Chordata (Tunicata), Porifera, Annelida, Bryozoa, Cnidaria, Arthropoda, Mollusca, Echinodermata, Rhodophyceae, Chlorophyceae, Phaeophyceae (Fig. 4.19). Species other than oysters were not recorded as part of this study. The fouling communities varied between the locations; these observations will be addressed by additional ongoing work (Harris-Scott *et al.* unpublished data). Settlement of both *O. edulis* and *C. gigas* was only observed when the presence of other fouling species was minimal and no evidence of prior settlement beneath or amongst these communities was observed. There was also no evidence of predation upon any settled spat of either species.

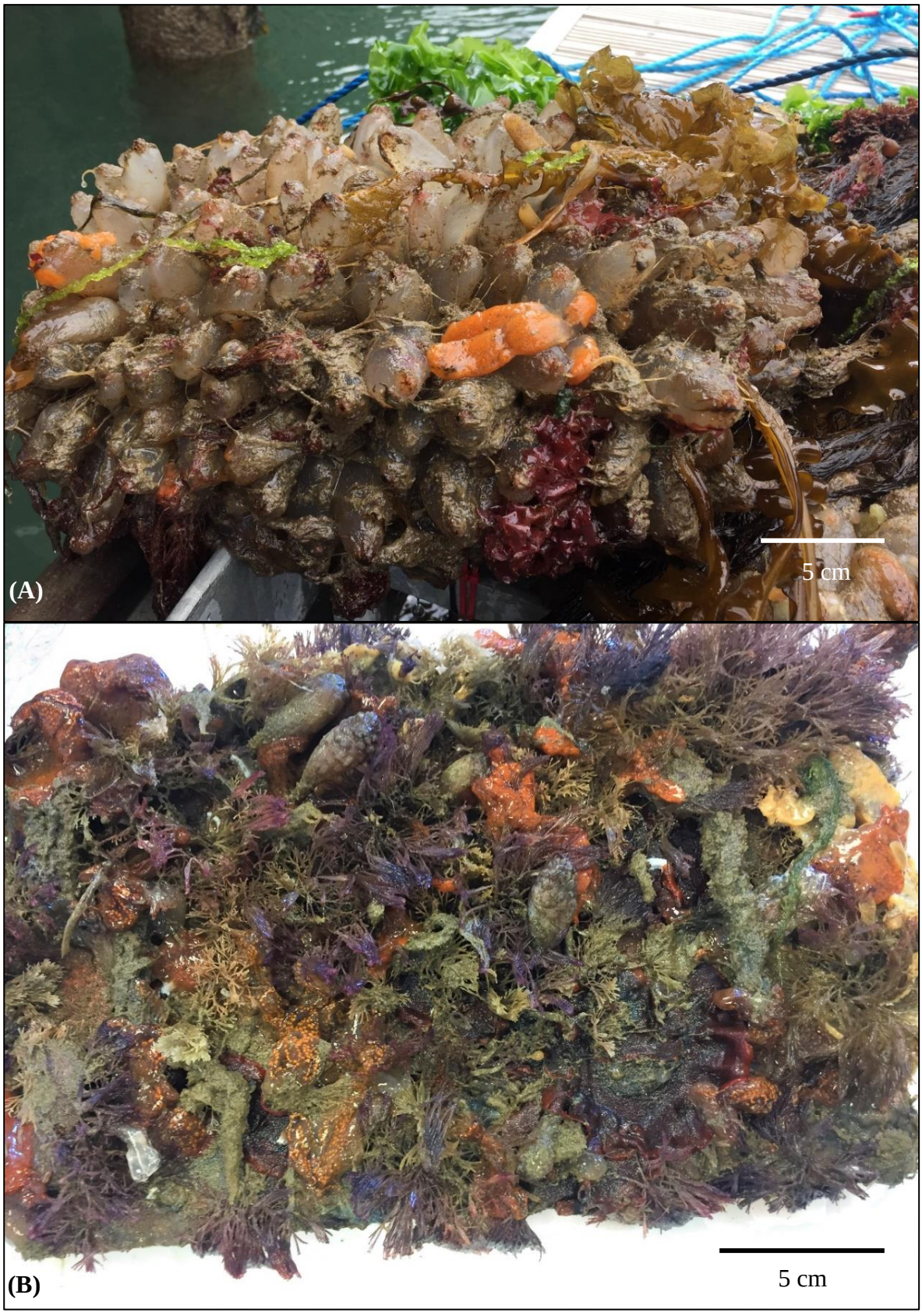


Figure 4.19. Fouling community observed on (A) a horizontally oriented settlement plate deployed in Portsmouth Harbour (BA) and (B) the underside of a horizontally oriented settlement plate deployed in Sparkes Marina, Chichester Harbour (SP). Tile dimensions: 27 x 16.5 x 1 cm.

4.4. Discussion

4.4.1. Brooding and fecundity

Understanding the reproductive activities of *Ostrea edulis* in restoration projects is of utmost importance and key to recurrent recruitment success. The European flat oyster is notoriously sporadic with regards to spawning activity and successful spatfall events, thus determining the approximate timings and duration of breeding activity allows for management practices to be organised appropriately. Historic data sets for the intended restoration areas can provide an invaluable insight into temporal changes in such behaviours, for example Utting *et al.* (1991) observed the main brooding activity within the Solent to occur between July and September during the 1985 - 1987 seasons. Monitoring was previously not conducted earlier in the season, during May or June, possibly due to the knowledge at the time that brooding did not occur that early in the year. However, this is unlikely as the results presented in this study indicate that the peak in brooding activity for 2017 occurred during June into July with the "tail end" of activity extending into August. The results of this study are identical to those presented by Korringa (1957) whose long-term observations, conducted daily or twice daily over 20 complete seasons, show the height of the spawning season is between the last ten days of June and the first ten days of August.

When considering the correlation between breeding and water temperature throughout the geographical range of *O. edulis* (Orton, 1920) it could have been expected that raising oysters from the seabed to the surface of the water column, in the manner they were in this study, would induce the onset of reproduction earlier in the year. As can be seen from the results, this was not the case and is again in agreement with the findings of Korringa (1957) and Waugh (1952, 1953, 1954, 1955). Although, in agreement with Orton (1920), spawning did not occur before water temperatures reached 15 - 16°C, spawning was not

instantaneously induced upon reaching this temperature and did not continue into September or October despite the temperature remaining above 15 °C. The results from this study further support the statement that the correlation cannot be a very close one and that in fact there is a stronger correlation to lunar, thus tidal, state (Korringa, 1947, 1957). Despite the lack of daily monitoring in this study prior to the sampling period, the dates where the greatest peak in brooding activity occurs are also identical between this study and Korringa's. The proportion of the total adult population observed spawning during the 2017 season also, encouragingly, correlates with, and marginally exceeds, the reported figures of 10 - 20 % in cultured settings (Walne 1974).

Alongside the occurrence and duration of the spawning season, the quantity of larvae within each brood is of extreme importance. There are many reports on the fecundity of *O. edulis* dating back to that of Eyton (1858) who estimated very larger oysters could produce 1,800,000 larvae, a figure not too dissimilar to those obtained in this study. Other historical reports range from 95,000 - 1,730,200 in oysters that are between one and four years old (Moebius, 1883; Buckland quoted in Philpots, 1890; Dantan, 1913; Gaarder and Bjerkan, 1934; Orton, 1937 in Cole, 1941; Walne, 1964; Utting, 1991). With Gerbe (1876) the first to describe egg production during the first year, post settlement.

Cole (1941), Walne (1964) and Utting *et al.* (1991) all observed considerable variation in the fecundity of oysters of a similar size, however, all concluded a positive correlation between brood size and adult size, with Walne (1964) additionally showing this correlation with adult size rather than age. Similar variability was also observed in *O. lurida* with broods ranging from 69,490 - 355,500 larvae (Hopkins, 1937). Oysters collected in this study also displayed considerable variation, up to 736,783 larvae, in fecundity between oysters of similar sizes for measurements of maximum shell length, width depth and whole wet weight.

The larval brood size also showed significant positive correlations with maximum shell length, maximum shell depth and whole wet weight.

In contrast to the results presented in this study, Utting *et al.* (1991) did not observe significant differences in fecundity between locations within the Solent, suggesting perhaps that oysters established in their native areas across a relatively small spatial scale do indeed produce similar brood sizes. It is clear from the results of this study that the oysters translocated from the same source location were influenced by the environmental conditions in the areas that they were moved to, altering the occurrence of brooding and the capacity for the quantity of larvae they could brood. Epigenetic changes to larval brood size appear to be driven by environmental factors, particularly temperature and turbidity. The oysters translocated to areas closest to the source location produced greater quantities of larvae and brooding occurred in oysters over a greater size range. From the parameters available in the Environment Agency archive dissolved oxygen and temperature were shown to be the greatest contributing factors to the occurrence of brooding and the quantity of larvae produced. The variations in the areas of the central Solent may have contributed to the reduced brooding activity and the peak occurring in July rather than June as with the eastern populations. This alteration in the environmental conditions may have meant that those oysters in areas closest to the source location were better adapted to surrounding conditions and therefore in better condition earlier in the season, thus able to allocate a larger amount of energy to gonadal growth and egg production in comparison to those in the central Solent areas.

Walne (1964) determined that an oyster in good condition have the capacity for 50 % greater egg production to an equivalent sized individual in poor condition. Cole (1941) suggests that oysters maturing as females towards the final few months of the season, after being in the male phase for the months prior to this, are likely to produce considerably fewer

larvae than those entering the season as females. The survival of the larvae may also depend on source location as has been observed with *C. virginica* (Eierman and Hare, 2013).

The stocking desnity was also shown to influence broodsize, with those adults held at the half-density likely to have experienced lower levels of biofouling, leading to them receiving a greater flow of water into the cages, thus improved food supply. This would likely have provided them with greater energy reserves to produce more larvae with greater lipid reserves themselves. If this were the case, then it is also beneficial for the longer-term survival of recruits (Araya *et al.*, 2012; Roberts *et al.*, 2017).

From the results obtained in this study it is clear that the preservation of larvae in ethanol, thus dehydration, alters the size of the larvae from the expected size ranges and alternative sample preparation techniques should be considered for future sample analysis. Therefore, the development and condition of the larvae brooded at the various locations cannot be commented on with confidence. Although not in the financial and logistical remit of this study, it will be important to determine chlorophyll-a concentrations, as a proxy for food availability, in future studies to confirm if this increase in larval production, and size of larvae, seen within areas closest to the source location is indeed attributed to this factor.
From an initial stocking density of approximately 10,000 oysters across six locations, the total number of larvae estimated to have been produced and brooded during the 2017 season was 1,174,668,944. This quantity of larval production, under favourable settlement conditions with suitable quantities of available substrata, may result in the settlement and development of 2,349 spat, to at least one year of age, when estimating from reported success of two survivors from one million larvae (Guerra, 2002 in Laing *et al.*, 2005) or 117,466,894 when accounting for survival rates of 10 % seen in hatchery environments (Walner, 1974).

Fisheries management should accommodate sustainable removal of the target species in a manner that maximises the number of offspring produced. The current regulations for

the Southern IFCA and Sussex IFCA districts minimum landing size (MLS), for *O. edulis*, state that no oysters that can pass through a circular ring with an internal diameter of 70 mm should be removed (Southern IFCA 2018; Sussex IFCA 2018). The results of this study show that fecundity is correlated with maximum shell length and depth, and whole wet weight, and suggest that a MLS of 70 mm in any direction is likely to be insufficient to allow for the oysters to reach a size whereby they have contributed a significant amount of larvae to the population for a number of consecutive years. When using the data from Walne (1974), along side the information from this study, it can be shown that by increasing the MSL oysters will reproduce for several years, increasing in numbers each time. For example, with the assumption that the individual reproduces each year, if an oyster is removed at approximately 60 mm (~ 2 years old), it will likely have contributed 640,000 larvae to the system, if removed at 70 mm (~ 3 years old) it will have contributed 1,480,000 larvae to the system and if removed at 80 mm (~ 4 years old) it will have contributed 2,580,000 larvae to the system. It is therefore suggested that if the fisheries re-open in the future, any oysters landed should be presented against a rectangular device with internal dimensions measuring 30 mm for maximum shell depth and 75 - 80 mm for maximum shell length, as opposed to the current 70 mm ring (Fig. 4.20). It is unrealistic to suggest that all landed oysters be weighed at sea for fisheries purposes, as whole wet weight is strongly correlated with maximum shell length and maximum shell depth it is also likely to be unnecessary.

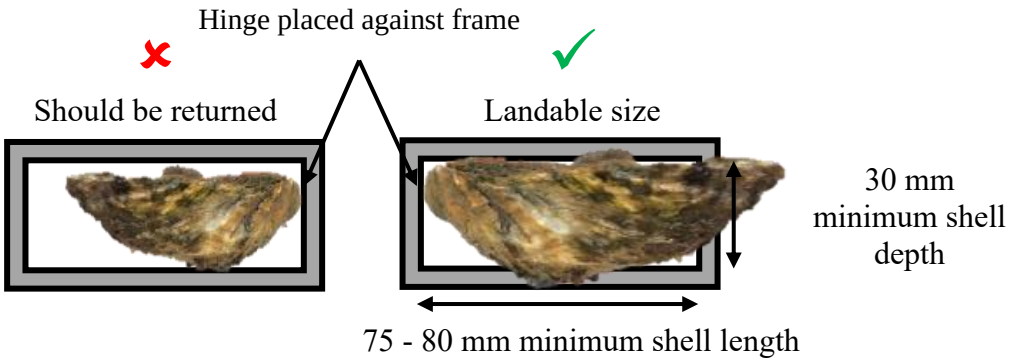


Figure 4.20. Suggested management approach to minimum landing size for *Ostrea edulis* in the Solent, incorporating the Southern and Sussex Inshore Fisheries and Conservation Authority districts. Rectangular measuring device with internal dimensions of 30 mm x 75 - 80 mm as opposed to circular ring with internal diameter of 70 mm.

Incorporating shell depth into require measurements is likely to increase the period before an individual can be removed from the fishery, as this direction of growth occurs over a longer period with new growth fringes primarily occurring posteriorly prior to thickening. By implementing such management measures, oysters would be provided with the time necessary to successfully settle, reach a size and age whereby they are likely to have reproduced in excess of three times, substantially increasing their fecundity each time, and contributing sufficiently to the reproduction of the species (Cole, 1941) before being legally landed. This will allow for an increase in the number of recruits per spawner and at this suggested size the probability of the adults being able to provide the settlement surface needed by the offspring increases, the recuits could be then also returned upon landing. This long-term approach to fishery management is going to be essential to allow sufficient quantities of oysters to reach a population size whereby it becomes self-sustaining and where sustainable removal of limited stock will be allowable. Increasing the MLS will also mitigate against the removal of substantial numbers of currently undersized oysters, < 70 mm, which have been landed in recent years, see Chapter 2, and that are likely to have been landed historically.

4.4.2. Larval settlement

When taking into account the relatively small quantities of broodstock and dimensions of the settlement plates used in this study, alongside the enormity of the Solent water body, total available substrata for larval settlement, and the settlement behaviours towards the benthos, it can be considered encouraging that *O. edulis* spat were indeed observed settling near the surface of the water column. The presence of a greater number of *Crassostrea gigas* spat on the settlement plates is likely to reflect the influence from a variety of factors. The most prominent of which is the presence of a greater abundance of mature *C. gigas* in the environments where the plates were deployed. The oviparous nature of their reproduction and relatively early sexual maturity along with the ability of those adults to produce substantial numbers of sperm and eggs, several millions (Walne, 1974; Guo and Allen, 1997; Kang *et al*., 2003), undoubtedly increases the likelihood of successful settlement of such a substantial population.

Rapid growth and a high survival rate of those settled *C. gigas* larvae (Diederich, 2006), in comparison with those of *O. edulis* (Askew, 1972; Pogoda *et al*., 2011; Otero *et al.*, 2103), is likely to have allows them to accommodate competition from other macroinvertebrate species settling at a similar period in time. The number of observed settled spat may have been greater if the removal of the 2018 plates had taken place two to three months prior, as with the 2017 plates, before the non-target fouling community had fully established.

In addition, the rapid growth of *C. gigas* will have allowed for reduced mortality that would have arisen from sediment smothering on the upper surface of the horizontal plates in the slower growing *O. edulis* that may have settled. However, Cole and Knight Jones (1939) also determined that the underside of horizontal surfaces are still preferentially selected by *O. edulis* when the limitations presented by silt are removed. Even with high settlement

success the mortality of spat within the first autumn and winter can exceed 90 % (Cole, 1951). Settlement of *O. edulis* in this study did not occur in great enough quantities for any comments to be made on orientation preference with confidence. The presence of fewer *O. edulis* highlights the need for a larger broodstock population that is provided with the necessary protection from dredging activities and that can provide a source of larvae to both protected and managed fishery areas. In addition, these results highlights the need for the deployment of supplementary substratum, at an appropriate time prior to larval release, in areas where larvae are predicted to accumulate, and where populations thrived historically as the settlement of *O. edulis* larvae has been shown to be determined by the availability of hard substrata (Smyth *et al.*, 2018). Further investigations should be conducted to determine how the settlement of both *O. edulis* and *C. gigas* would vary if similar experiments were trialled on the seabed. The severity of fouling experienced in this study, with regards to tunicate and algal species, is likely to be higher due to the food availability and light intensity at the surface of the water column.

Once settlement of an initial cohort occurs then the presence of these conspecifics will encourage the settlement of further cohorts allowing for multiple year classes to be present within the population (Rodriguez-Perez *et al.*, 2019). If making the comparison to terrestrial farming practices, those working the land will sow seeds, harvest the crop and re-sow seed, even fertilising the land to encourage growth and taking measures to remove pests. Whereas, for centuries *O. edulis* populations in the Solent have simply been harvested as a crop under the impression that they are inexhaustible, evidently not the case. As Cole (1951) expressed, the necessity to remove pests, cultivate intensively, clean cultch and sow spat on shell and / or broodstock oysters to encourage gregarious settlement (Bayne, 1969) has been well understood and expressed as "*imperfectly recognised*" prior to the 1950s, leading to much wasted effort of restocking attempts and therefore, fishery practice.

Increases in the abundance and spatial distribution of *C. gigas* populations in the Solent, shown further by the number of spat observed between 2017 and 2018, are likely to reflect increasingly favourable environmental conditions that are accommodating large-scale reproduction and spatfalls of this species, originally introduced under the impression that the conditions were too cold for reproduction to occur. The presence of *C. gigas* in areas where *O. edulis* was once abundant is contentious with both beneficial and detrimental impacts shown for biodiversity (Zwerschke *et al.*, 2016; Guy *et al.*, 2018), interspecific competition for food and space with native *Mytilus edulis* (DAISIE, 2013) as well as settlement of *O. edulis* (Christianen *et al.*, 2018). Co-occurrence of *O. edulis* and *C. gigas* in intertidal habitats has been documented in Ireland (Zwerschke *et al.*, 2018) and observed in multiple locations within the Solent (pers. obs.) indicating that at present there is the capacity for populations of both species to mutually benefit from increased settlement.

The results presented highlight the efficiency of broodstock cages as larval pumps, with significant improvements in brood size when stocked at the lower density of 60 oysters per cage. The estimated output of each cage per year can be calculated from the obtained values as:

Average number of brooding adults = 4.8 % of 60 oysters = 3;

Average brood size = 1,312,633 *3 x 1,312,633 = 3,937,899 larvae per cage*

Survival, therefore potential recruitment, to one year of age could be between 8 - 393,789 spat per cage when estimating from reported success of two survivors from one million larvae or 10 % survival seen in hatcheries (Walner, 1974; Guerra, 2002 in Laing *et al.*, 2005) and further highlights the need for suitable settlement substrata.

Chapter 5

The Efficacy of Suspended Broodstock Cages as a Restoration Strategy - Disease

Published in part as: Helmer, L. Hauton, C., Bean, T., Bass, D., Hendy, I., Harris-Scott, E., & Preston, J. (2019). Ephemeral detection of *Bonamia exitiosa* (Haplosporidia) in adult and larval European flat oysters *Ostrea edulis* in the Solent (UK). *Journal of Invertebrate Pathology*.

5.1. Introduction

Protozoan parasites within the orders Haplosporidia and Paramyxida are the causative agents of bonamiosis and marteiliosis, which have been discovered to be responsible for numerous mortality events within populations of not only *Ostrea edulis* (Grizel *et al*., 1974), but many other bivalve species across the globe (Perkins & Wolf, 1976; Cranfield *et al*., 2005; Culloty & Mulcahy, 2007; Engelsma *et al*., 2014; Kerr *et al*. 2018).

Eradication of all susceptible and vector species for these pathogens in the marine environment is impossible, therefore, the best preventative measure is to monitor and control the movement of positive populations known to be carrying the pathogens. The threat of these pathogens to naïve populations of host species across Europe is so severe that *Bonamia ostreae, Bonamia exitiosa* and *Marteilia refringens* have all been recognised by both the World Organization for Animal Health (OIE) and the European Union (under EC Directive 2006 + 88) as a significant pathogen of bivalve molluscs (OIE, 2019). Routine surveillance and testing programmes allow for decisions regarding the movement of oyster stocks between sites. Traditional diagnostic techniques, such as histology and cytology, involve the preparation and staining of gill or heart tissue for visualisation by light microscopy. In recent years the development of molecular techniques such as polymerase chain reaction (PCR) have enabled the detection of pathogen DNA within the host tissue, providing a faster and more sensitive detection. A combination of both techniques enables the highest level of certainty when assessing infection.

This section of the study details the current status of *B. ostreae* and *B. exitiosa* within populations of *O. edulis* across the Solent using molecular techniques. The efficacy of three commonly used primer pairs for the detection of *B. ostreae* were also analysed, two of which are recommended by the OIE.

5.2. Methods

5.2.1. Presence of *Bonamia ostreae* and *Bonamia exitiosa* within broodstock populations and their broods

5.2.1.1. Mature oyster provenance

All oysters used to stock the broodstock cage populations in 2017 and 2018 were obtained during November 2016 from Langstone Harbour and were tested for the presence of *B. ostreae* by Centre for Environment Fisheries and Aquaculture Science (CEFAS), as previously mentioned in 2.2.5. The histological analysis with molecular conformation with the use of the primer pair BO + BOAS (Cochennec *et al*., 2000), recorded a *B. ostreae* presence within 4.1 % the population and provided the baseline reading before the broodstock cage trial commenced. Samples were collected and then preserved, from all locations after one (October 2017) and two (November 2018) years within the broodstock cage systems. For testing of each specimen, a 5 mm of gill tissue and the whole heart organ were preserved by placing them into 70 % ethanol within a 1.5 ml Eppendorf tube to be stored at -20 ℃ prior to DNA extraction in 2019.

The gill and heart tissue from each sample was thoroughly macerated with a sterile scalpel or pellet pestle. DNA extractions were performed using DNeasy® Blood & Tissue kits (QIAGEN™, Germany) following the manufacturer's tissue protocol. Quantification of the DNA extractions was conducted using a NanoDrop® 1000 Spectrophotometer (NanoDrop®, Thermo Fisher Scientific Inc., USA).

Species-specific primer pairs were used to amplify *O. edulis, B. ostreae, B. exitiosa* and *C. gigas* DNA (Table 5.1). In addition to the samples from the 2017 and 2018 broodstock trials, DNA extracted from the Portsmouth Harbour and Chichester Harbour seabed fishery populations collected in 2015 (Chapter 2, also in Helmer *et al*., 2019) was also analysed further with the additional use of the BO + BOAS, BOSTRE and BEXIT primer pairs (Table

5.1). The 2015 seabed oysters were used to stock broodstock cages in Portsmouth and Langstone harbours until July 2016, a proportion of these were also preserved, prepared, and analysed as previously mentioned. For both the 2015 seabed and 2016 broodstock cage populations, only the gill tissue was used for extractions and was prepared as previously described.

Table 5.1. Primer sequences used in this study. The Oe fw_1 + Oe rev_4 (Gercken & Schmidt, 2014) primer pair used to amplify *Ostrea edulis* DNA, BO + BOAS (Cochennec *et al*., 2000), C_F + C_R (Carnegie *et al*., 2000) and BOSTRE-F + BOSTRE-R (Ramilo *et al*., 2013) used to amplify *Bonamia ostreae* DNA, BEXIT-F + BEXIT-R (Ramilo *et al*., 2013) used to amplify *Bonamia exitiosa* DNA and LCO1490 + HCO2198 (Folmer *et al*., 1994) used to amplify invertebrate metazoan DNA, including *Crassostrea gigas*.

Species	Primer pair	Primer sequences	Target gene	Amplicon size (bp)	Reference
Ostrea edulis	Oe fw_1	5'-ATG-GGA-CGA-TTT-GAT-AGA-GC-3'	*coxI*	1100	(Gercken & Schmidt, 2014)
	Oe rev_4	5'-CCC-AAA-TAA-CGG-GAA-AAG-TGC-TAA-CCA-CCA-GAA-TGA-3'			
Bonamia ostreae	BOSTRE-F	5'-TTA-CGT-CCC-TGC-CCT-TTG-TA-3'	18S ITS 1	208	(Ramilo *et al.*, 2013)
	BOSTRE-R	5'-TCG-CGG-TTG-AAT-TTT-ATC-GT -3'			
Bonamia ostreae	BO	5'-CAT-TTA-ATT-GGT-CGG-GCC-GC-3'	SSU	300	(Cochennec *et al.*, 2000)
	BOAS	5'-CTG-ATC-GTC-TTC-GAT-CCC-CC-3'			
Bonamia ostreae	C_F	5'-CGG-GGG-CAT-AAT-TCA-GGA-AC-3'	18S-ITS 1	760	(Carnegie *et al.*, 2000)
	C_R	5'-CCA-TCT-GCT-GGA-GAC-ACA-G-3'			
Bonamia exitiosa	BEXIT-F	5'-GCG-CGT-TCT-TAG-AAG-CTT-TG-3'	18S ITS 1	246	(Ramilo *et al.*, 2013)
	BEXIT-R	5'-AAG-ATT-GAT-GTC-GGC-ATG-TCT-3'			
Diverse metazoan invertebrates (*Crassostrea gigas*)	LCO1490	5'-GGT-CAA-CAA-ATC-ATA-AAG-ATA-TTG-G-3'	*coxI*	~ 650	(Folmer *et al.*, 1994)
	HCO2198	5'-TAA-ACT-TCA-GGG-TGA-CCA-AAA-AAT-CA-3'			

Polymerase chain reaction (PCR) amplifications were all conducted in a final volume of 25 μl, consisting of 12.5 μl 2 x DreamTaq™ PCR Master Mix (Thermo Fisher Scientific Inc.) or 12.5 μl 2 x DreamTaq Green PCR Master Mix (Thermo Fisher Scientific Inc.), 0.2 μM forward and reverse primers (Invitrogen), 1 - 5 μl extracted total DNA (20 - 200 ng), and 6.5 - 10.5 μl molecular biology grade water (Thermo Fisher Scientific Inc.)

A blank sample containing either 12.5 μl 2 x DreamTaq™ PCR Master Mix (Thermo Fisher Scientific Inc.) or 12.5 μl 2 x DreamTaq Green PCR Master Mix (Thermo Fisher Scientific Inc.), 0.2 μM forward and reverse primers (Invitrogen) and 11.5 μl molecular biology grade water was run with each set of samples as a negative control.

The PCR program ran in a G-STORM 482 - 48 Well Multi Block Thermal Cycler (Gene Technologies Ltd., England) under the conditions in Table 5.2.

Table 5.2. Polymerase chain reaction conditions for the primer pairs Oe fw_1 + Oe rev_4 used to amplify *Ostrea edulis* DNA, BO + BOAS, CF + CR and BOSTRE-F + BOSTRE-R used to amplify *Bonamia ostreae* DNA, BEXIT-F + BEXIT-R used to amplify *Bonamia exitiosa* DNA and LCO1490 + HCO2198 used to amplify *Crassostrea gigas* DNA.

		35 cycles			
Primer pair	**Initial denaturation**	**Denaturation**	**Annealing**	**Extension**	**Final extension**
Oe fw_1 Oe rev_4	5 min 94 °C	60 s 94 °C	60s 45°C	60s 72 °C	10 min 72 °C
BO BOAS	5 min 94 °C	60 s 94 °C	60s 55°C	60s 72 °C	10 min 72 °C
C_F C_R	5 min 94 °C	60 s 94 °C	60s 55°C	60s 72 °C	10 min 72 °C
BOSTRE-F BOSTRE-R	2 min 94 °C	30 s 94 °C	45s 55°C	60s 72 °C	1 min 72 °C
BEXIT-F BEXIT-R	2 min 94 °C	30 s 94 °C	45s 58°C	60s 72 °C	1 min 72 °C
LCO1490 HCO2198	5 min 94 °C	60 s 94 °C	60s 45°C	60s 72 °C	10 min 72 °C

Resulting PCR products from the Oe fw_1 + Oe rev_4, C_F + C_R and LCO1490 + HCO2198 primer pairs were run on 1 % agarose (Fisher Scientific, UK) gel composed of 100 ml 1X Tris-acetate-EDTA (TAE) buffer and 4 µl ethidium bromide (Sigma-Aldrich®). A 1 kb DNA ladder (Thermo Fisher Scientific Inc.) was used as a reference for the Oe fw_1 + Oe rev_4 and LCO1490 + HCO2198 primer pair products, whilst a 100bp DNA ladder (Fisher Scientific, New England Biolabs®Inc. or PCR Biosystems) was used as a reference for the C_F + C_R primer pair products.

PCR products from the BO + BOAS, BOSTRE and BEXIT primer pairs were run on 2 % agarose (Fisher Scientific, UK) gel composed of 100 ml 1X TAE buffer and 4 µl ethidium bromide (Sigma-Aldrich®). A 100 bp DNA ladder (Fisher Scientific, New England Biolabs®Inc. or PCR Biosystems) was used as a reference. Electrophoresis was performed at 100 V for 1 h, following this the samples were visualised by ultraviolet (UV) transillumination in a 'VWR® Gel Documentation Smart Version system'.

A positive reference sample from each primer pair were selected from each year's samples and all positive amplifications using the BEXIT primer pair were selected. Purification of positive reference samples from all primer pairs was conducted using a QIAquick® PCR Purification Kit (QIAGEN™, Germany) following the protocol provided by the manufacturer. Purified PCR products were sent to Source BioScience (Nottingham, England) for Sanger sequencing, and the chromatograms were analysed using the programme MEGA X: Molecular Evolutionary Genetics Analysis, v. X (Kumar *et al.*, 2018). All sequences generated were searched for similarity using the nucleotide Basic Local Alignment Search Tool, BLASTn (National Center for Biotechnology Information, USA). See Appendix E for details on samples selected for purification and sequencing.

5.2.1.2. Brooded larvae

Larvae were collected prior to fecundity analysis, as explained in 4.2.2. A small proportion (500 µl) of the pooled larvae from each brooding adult were removed, placed into a 1.5 ml Eppendorf Tube® and immersed in absolute ethanol for molecular analysis to determine the presence or absence of *B. ostreae* and or *B. exitiosa*. An aliquot of 250 µl of larvae was taken from each sample, unlike the gill and heart tissue of the mature oysters this required no additional mechanical breakdown prior to the DNA extraction process being performed as described in 5.2.1.1.

PCR and electrophoresis were conducted as described for the mature broodstock oyster samples in 5.2.1.1, with the exception that PCR analysis was not conducted using the C_F + C_R and LCO1490 + HCO2198 primer pairs. Where possible larval samples were then compared with the results of the individuals found to be brooding them.

Purified PCR products were sent to Source BioScience (Nottingham, England) for Sanger sequencing. Raw sequence chromatograms were checked by eye for quality and subsequently analysed using the software MEGA X: Molecular Evolutionary Genetics Analysis, v. X (Kumar *et al.*, 2018). All sequences generated were searched for similarity using Basic Local Alignment Search Tool (BLAST) through web servers of the US National Center for Biotechnology Information. See Appendix E for details on samples selected for purification and sequencing.

5.2.1.3. Phylogenetic analysis

Sequences of *B. exitiosa* obtained in this study were aligned with published small subunit (SSU) rRNA sequences with a wide geographic distribution across a number of oyster host species (Table 5.3). Alignment was carried out using the ClustalW setting in MEGA v. X (Kumar *et al.*, 2018) so that all sequences spanned identical regions. The data set was then realigned using the ClustalW setting. Phylogenetic trees were produced by Neighbor-Joining analysis of the sequence data using the p-distance method (Nei and Kumar, 2000). Gaps and missing data were only eliminated in pairwise sequence comparisons (pairwise deletion option). Bootstrap confidence intervals estimated were not reliable due to the limited phylogenetic information within the gene sequences. Maximum Likelihood phylogenetic analysis of the sequence data was also used applying the Tamura 3-parameter substitution model (Tamura, 1992). Standard error estimated obtained by using a bootstrap procedure were not reliable due to the limited phylogenetic variability available. Gaps and missing data were treated as complete deletion.

Table 5.3. GenBank accession numbers of the *Bonamia* spp. small subunit rRNA sequences used in the alignments for subsequent phylogenetic and distance analysis. Sequences published within Carnegie *et al.* (2000), Hill *et al.* (2010) and Hill *et al.* (2014).

Bonamia species	Sampling location	Host species	GenBank accession nos.
B. exitiosa	Australia	*Saccostrea glomerata*	JF831683
B. exitiosa	New Zealand	*Ostrea chilensis*	JF831655
B. exitiosa	California, USA	*O. conchaphila*	JF831723
B. exitiosa	North Carolina, USA	*O. stentina*	JF831588
B. exitiosa	South Carolina, USA	*O. stentina*	JF831599
B. exitiosa	Argentina	*O. stentina*	JF831573
B. exitiosa	Argentina	*O. puelchana*	JF831633
B. exitiosa	New Zealand	*O. stentina*	JF831661
B. exitiosa	Florida, USA	*Crassostrea ariakensis*	JF712867
B. sp. ex	Tunisia	*O. stentina*	GU356032
B. ostreae	Maine, USA	*O. edulis*	AF162087

5.3. Results

5.3.1. Presence of *Bonamia ostreae* and *Bonamia exitiosa*

5.3.1.1. Total broodstock populations

All DNA extractions were successful and of the 346 broodstock oysters tested from 2015 - 2018, 99.7 % (345 / 346) provided positive amplifications for *O. edulis* DNA at 1,100 bp using the Oe_fw1 + Oe_rev4 primers. The one oyster that did not amplify *O. edulis* DNA was that which was visually identified as *Crassostrea gigas* and provided a positive amplification at ~ 650 bp using the LCO1490 + HCO2198 primer pair, the results of the BLAST search provided further positive identification for *Crassostrea gigas* with 99.69 % identity (KJ855245.1). Detection of *B. ostreae* DNA within the oysters sampled varied dramatically between primer sets. The BO + BOAS pair produced amplicons in 53.6 %, BOSTRE in 69.6 % and C_F + C_R in 15.9 % of the same sample set. *Bonamia exitiosa* DNA was detected in four oysters (1.2 %) using the BEXIT primer pair (Fig. 5.1 and Table 5.4).

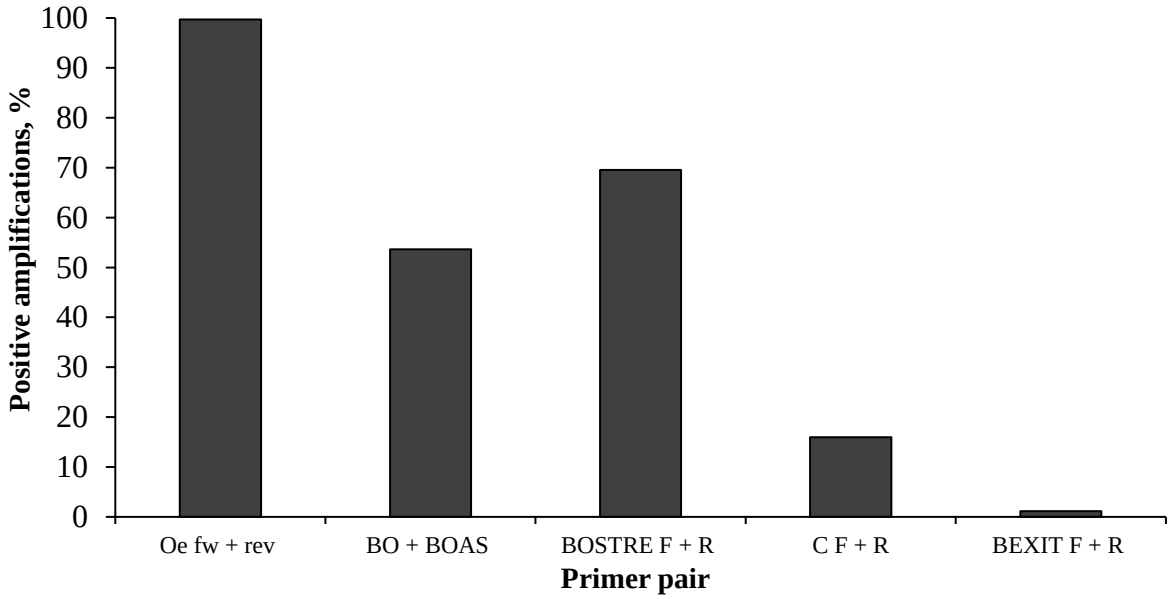


Figure 5.1. Percentage of broodstock oysters tested that provided positive results for *Ostrea edulis* DNA using the Oe_fw1 + Oe_rev4 primer pair (n = 346) and, of those individuals (n = 345), the percentage that provided positive results for *Bonamia ostreae* DNA using the BO+BOAS, BOSTRE-F+BOSTRE-R and C_F+C_R primer pairs, and *Bonamia exitiosa* DNA using the BEXIT-F+BEXIT-R primer pair from all broodstock samples in all locations across all years.

Presence of *B. ostreae* DNA did not vary greatly, with any of the three primers used, between the 2015 seabed and 2016 broodstock cage populations. An increase of 0.1 and 2.7 % was observed with the BO + BOAS and $C_F + C_R$ primer pairs, respectively. In comparison, a decrease of 2.4 % was observed with the BOSTRE primer pair. *Bonamia exitiosa* was detected in one individual from the 2015 seabed population (Fig. 5.2A).

The 2017 broodstock population experienced the highest level of detection across all sampling years for all three primer pairs. The initial presence in 4.1 % (sample n = 150) of the 2016 fishery population, obtained by CEFAS using histology and conformation using the BO + BOAS primer pair, was found to have increased by 79.6, 93.9 and 27.5 % with use of the BO + BOAS, BOSTRE and $C_F + C_R$ primer pairs, respectively, in the 2017 broodstock oysters. *Bonamia exitiosa* was detected in 3.1 % of the 2017 broodstock. This increase in the population found with the pathogen present did not continue into 2018 and a decrease in percentage of oysters containing *B. ostreae* DNA was observed, again with all three primer pairs. Using the BO + BOAS primers presence decreased by 31.4 % to 54.3 %, using the BOSTRE primers a decrease of 16.6 % was observed and when using the $C_F + C_R$ primer pair a decrease of 20.2 % was observed. *Bonamia exitiosa* was not detected in the 2018 broodstock (Fig. 5.2B).

During the 2017 sampling, presence of *B. ostreae* DNA within the brooding individuals, selected from within the broodstock cages, was 42.8, 41.4 and 31.6 % lower than the broodstock cage population as a whole using the BO + BOAS, BOSTRE and $C_F + C_R$ primer pairs, respectively. *Bonamia exitiosa* was not detected in any of the 2017 brooding adults (Fig. 5.2C).

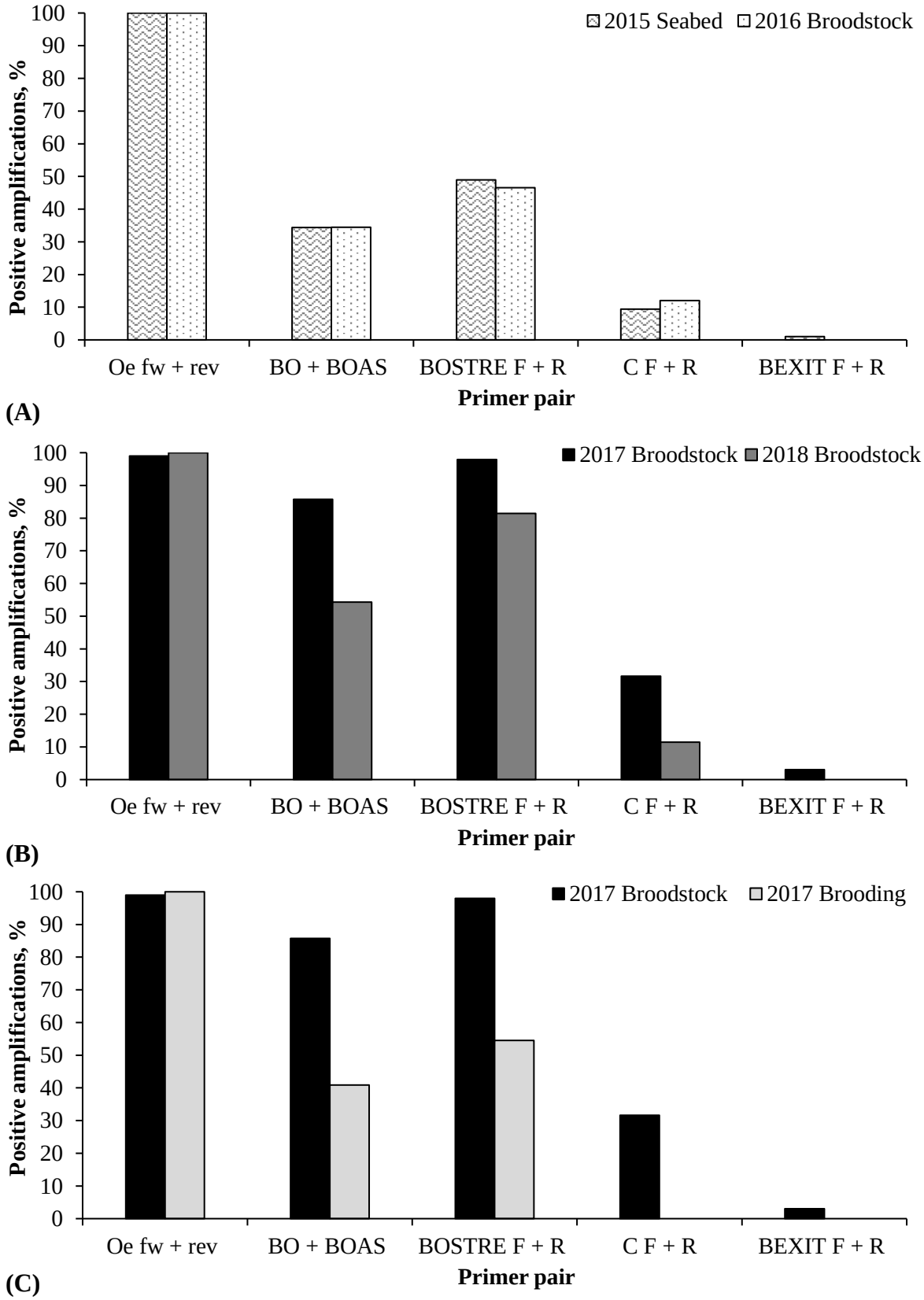


Figure 5.2. Positive amplifications of *Ostrea edulis* DNA (Oe_fw1+Oe_rev4), *Bonamia ostreae* DNA within *O. edulis* (BO+BOAS, BOSTRE-F+BOSTRE-R and C_F+C_R) and *Bonamia exitiosa* DNA within *O. edulis* (BEXIT-F+BEXIT-R) from (A) 2015 seabed (n = 96) and 2016 broodstock (n = 58), (B) 2017 broodstock (n = 99) and 2018 broodstock (n = 70), and (C) 2017 broodstock and 2017 brooding adults (n = 22).

Table 5.4. Primer sequences used in this section of the study to detect *Bonamia ostreae* and *Bonamia exitiosa* DNA within *Ostrea edulis* tissue from populations collected between 2015 and 2018. A total of 346 oysters were sampled, 345 of which provided positive amplifications for *O. edulis* DNA, thus total samples tested for *B. ostreae* and *B. exitiosa* are derived from the 345 confirmed to be native oysters. One individual was tested and confirmed to be *Crassostrea gigas*. An additional spat was analysed in 2017 from the broodstock cages and tested positive for *O. edulis* and *B. ostreae* but not *B. exitiosa*.

Species	Primer pair	Total		2015 Seabed		2016 Cages		2017 Cages		2017 Brooding		2018 Cages	
		+	-	+	-	+	-	+	-	+	-	+	-
Ostrea edulis	Oe fw_1 Oe rev_4	345	1	96	0	58	0	98	1	22	0	70	0
Bonamia ostreae	BO BOAS	185	161	33	63	20	38	84	15	9	13	38	32
Bonamia ostreae	BOSTRE-F BOSTRE-R	240	106	47	49	27	31	96	3	12	10	57	13
Bonamia ostreae	C_F C_R	55	291	9	87	7	51	31	68	0	22	8	62
Bonamia exitiosa	BEXIT-F BEXIT-R	4	342	1	95	0	58	3	96	0	22	0	70
Diverse metazoan invertebrates (*Crassostrea gigas*)	LCO1490 HCO2198	1	0	-	-	-	-	1	0	-	-	-	-

5.3.1.2. Larvae within brooding adults

All DNA extractions from 2017 and 2018 larval broods were successful and provided positive amplifications for *O. edulis* DNA at 1,100 bp using the Oe_fw1 + Oe_rev4 primers. Positive amplifications of *B. ostreae* DNA were detected in 64.5 and 77.4 % of the 2017 larval broods and 50 and 75 % of the 2018 larval broods using the BO + BOAS and BOSTRE primer pairs, respectively. Positive amplifications of *B. exitiosa* DNA were detected in 29 and 25 % of the larval broods tested from 2017 and 2018, respectively, using the BEXIT primer pair (Fig. 5.3A). The percentage of larval broods from 2017 was shown to be 23.6 and 22.9 % greater than the 2017 brooding population using the BO + BOAS and BOSTRE primer pairs, respectively (Fig. 5.3B).

A total of 21 brooding adults and their larval broods were suitable for comparative analysis and all provided positive amplifications for *O. edulis* DNA using the Oe_fw1 + Oe_rev4 primer pair. Presence of *B. ostreae* DNA was found both in the adults and their respective broods from six samples using the BO + BOAS primer pair and nine samples using the BOSTRE primer pair. When *B. ostreae* DNA was found to be absent from the brooding adults seven and six of the broods were found to contain the pathogen DNA using the BO + BOAS and BOSTRE primer pairs, respectively. *Bonamia ostreae* DNA was found to be present in three adults when it was absent from their broods using both the BO + BOAS and BOSTRE primer pairs. *Bonamia ostreae* DNA was not detected in either the adults or their broods in five and three samples using the BO + BOAS and BOSTRE primer pairs, respectively.

No brooding adult oysters were found to contain the DNA of *B. exitiosa*, neither were 16 of the broods. The broods of five oysters provided positive amplifications of *B. exitiosa* DNA at 246 bp using the BEXIT primer pair when their respective adult did not (Fig. 5.3C).

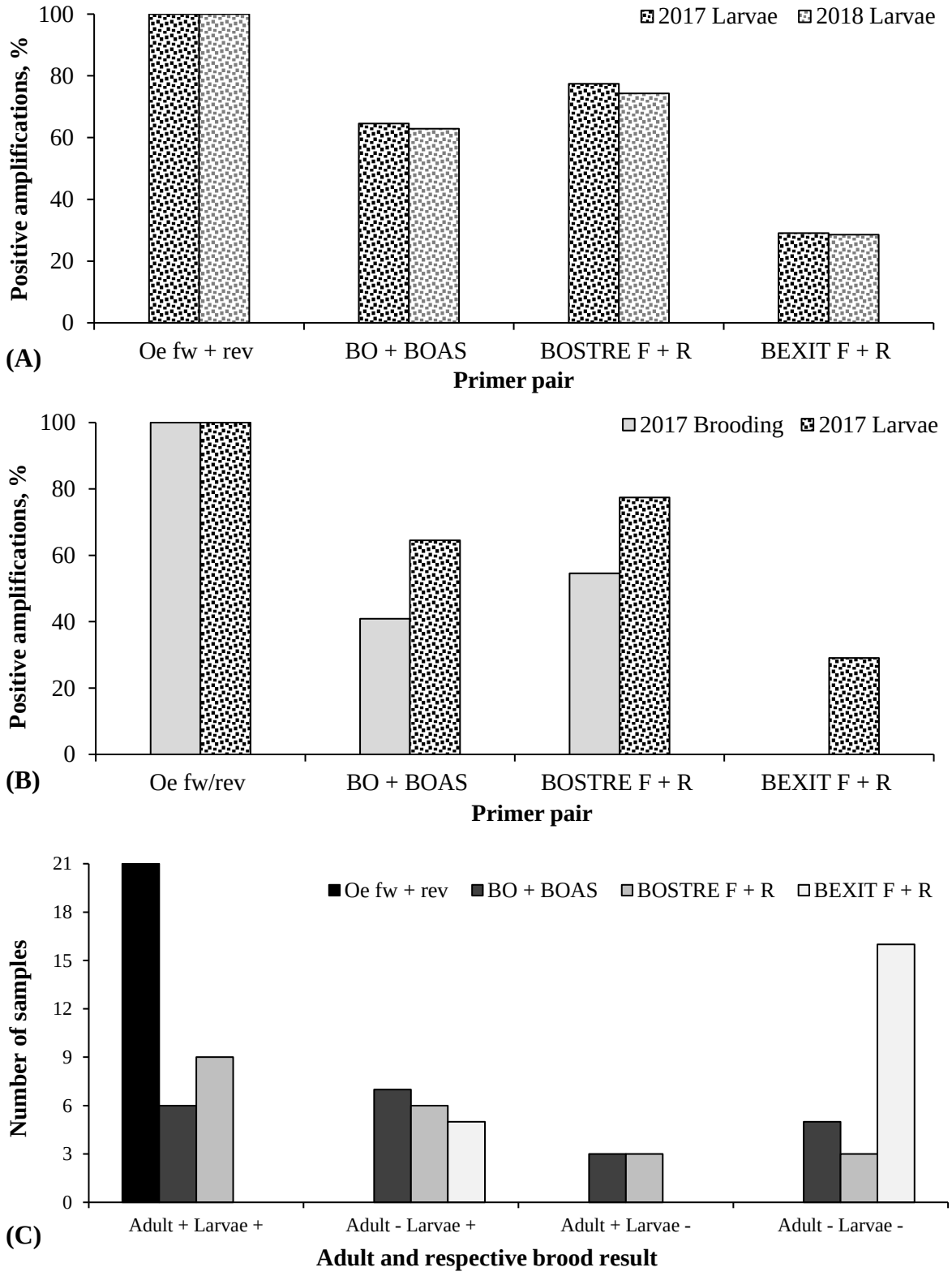


Figure 5.3. Percentage of oysters that provided positive results for *Ostrea edulis* DNA (Oe fw+rev primer pair), *Bonamia ostreae* DNA (BO+BOAS and BOSTRE F+R primer pairs), and *Bonamia exitiosa* DNA (BEXIT F+R primer pair) in (A) all 2017 (n = 31) and 2018 (n = 4) larval broods and (B) 2017 brooding oysters (n = 22) and 2017 larval broods. (C) The direct comparison of the positive or negative amplifications of *O. edulis*, *B. ostreae* and *B. exitiosa* DNA from individual brooding oysters and their respective larval brood.

The percentage of the 2017 brooding adult populations found to contain *B. ostreae* DNA was lower than that of the 2017 general broodstock population for all locations and all three primer pairs, with the exception of the Portsmouth Harbour population using the BOSTRE primer pair (5.9 % increase). The difference between the two populations varied from being 31.4 - 82.4, 33.3 - 88.2 and 17.6 - 52.9 % lower using the BO + BOAS, BOSTRE and C_F + C_R primer pairs, excluding the Portsmouth BOSTRE value. The percentage of the 2017 brooding adult populations found to contain *B. exitiosa* DNA was also lower than that of the 2017 general broodstock population for all locations. The difference between the two populations varied from 0 - 11.8 % using the BEXIT primer pair (Fig. 5.4).

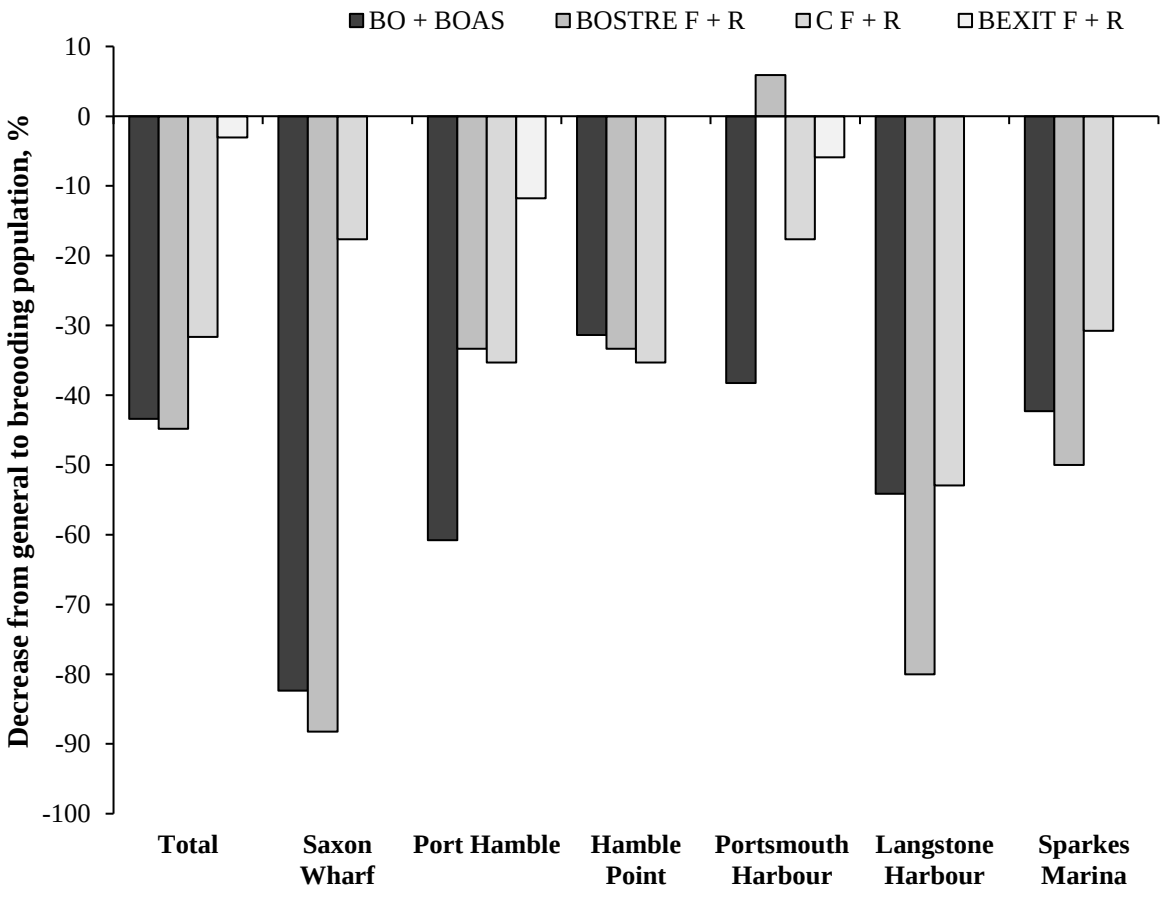


Figure 5.4. The difference in percentage between that of the general 2017 *Ostrea edulis* broodstock population (n = 99) to those found to be brooding in 2017 (n = 22) for the total and six individual marina populations found to contain *Bonamia ostreae* (BO + BOAS, BOSTRE-F+BOSTRE-R and C_F + C_R) and *Bonamia exitiosa* (BEXIT-F+BEXIT-R) DNA.

5.3.1.3. Impact of density, pressure-washing and position with cages on pathogen presence

The number of individuals containing *B. ostreae* DNA was slightly greater within the half-density populations from 2016 when compared to full-density populations using all three primer sets, with a difference of 2.2, 4 and 8.7 % shown with the BO + BOAS, BOSTRE and C_F + C_R pairs respectively (Fig. 5.5A). *Bonamia exitiosa* DNA was not detected in any of these individuals.

The number of individuals containing *B. ostreae* DNA within the half-density populations from 2017 was shown to be 2.3 % greater when using the BOSTRE primer pair, however, when using the BO + BOAS and C_F + C_R primer pairs the full-density populations were shown to have 4.8 and 12.8 % more infected oysters. *Bonamia exitiosa* DNA was only detected in oysters from half-density populations in this cohort (Fig. 5.5B).

The number of oysters containing *B. ostreae* DNA within the unwashed populations from 2018 was shown to be 10.6 and 14.7 % greater than those in the pressure-washed populations when using the BO + BOAS and C_F + C_R primer pairs, respectively. In contrast to this, the number of oysters containing *B. ostreae* DNA was 4.4 % fewer within the unwashed populations than those in the pressure-washed populations, when using the BOSTRE primer pair (Fig. 5.5C).

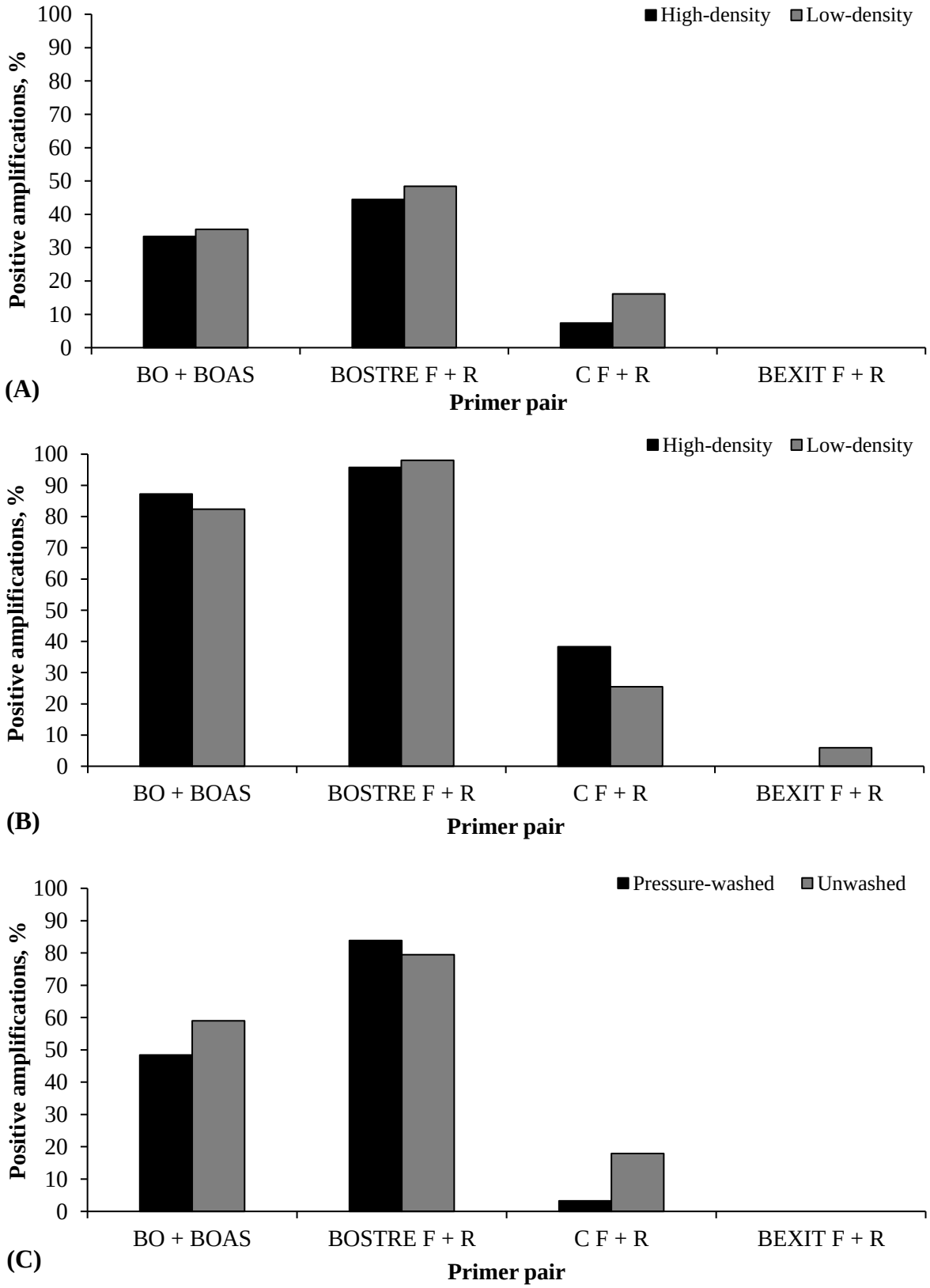


Figure 5.5. Presence of *Bonamia ostreae* (BO+BOAS, BOSTRE-F+BOSTRE-R and C_F+C_R) and *Bonamia exitiosa* (BEXIT-F+BEXIT-R) DNA within *Ostrea edulis* populations in (A) full- (n = 27) and half-density (n = 31) broodstock cages in July 2016, (B) full- (n = 47) and half-density (n = 51) broodstock cages in October 2017, and in (C) pressure-washed (n = 31) and unwashed (n = 39) broodstock cages in October / November 2018.

Pathogen presence with regards to *B. ostreae* was highest within the top micro-reef unit of the cages using the BO + BOAS, BOSTRE and C_F + C_R primer pairs for the pooled 2017 data, 87.5, 100 and 34.3 %, respectively. *Bonamia exitiosa* was only detected in the bottom micro-reef section of the cages in 2017 (9.8 %), using the BEXIT primer pair. The bottom micro-reef units within cages were shown to contain more oysters that tested positive for *B. ostreae* than those in the middle micro-reef units using the BO + BOAS and BOSTRE primer pairs, 84.4 % compared to 82.4 %, and 96.9 % compared to 94.1 %, respectively. Using the C_F + C_R primer pair showed a decrease in the number of oysters that tested positive for *B. ostreae* from the top (34.4 %) to middle (32.4 %), and from the middle to bottom (28.1 %) micro-reef unit (Fig. 5.6A).

The percentage of oysters within the 2018 broodstock cages, pooled from all locations, that tested positive for *B. ostreae* was shown to decrease from the top to middle, and from the middle to bottom micro-reef unit using the BOSTRE and C_F + C_R primer pairs, 85.7 to 83.3 to 73.7 % and 19 to 10 to 5.3 %, respectively. The number of oysters that tested positive using the BO + BOAS primer pair was greatest in the middle micro-reef unit, 56.7 %, and was marginally greater in the bottom micro-reef unit than the top unit, 52.6 and 52.4 % respectively (Fig. 5.6B).

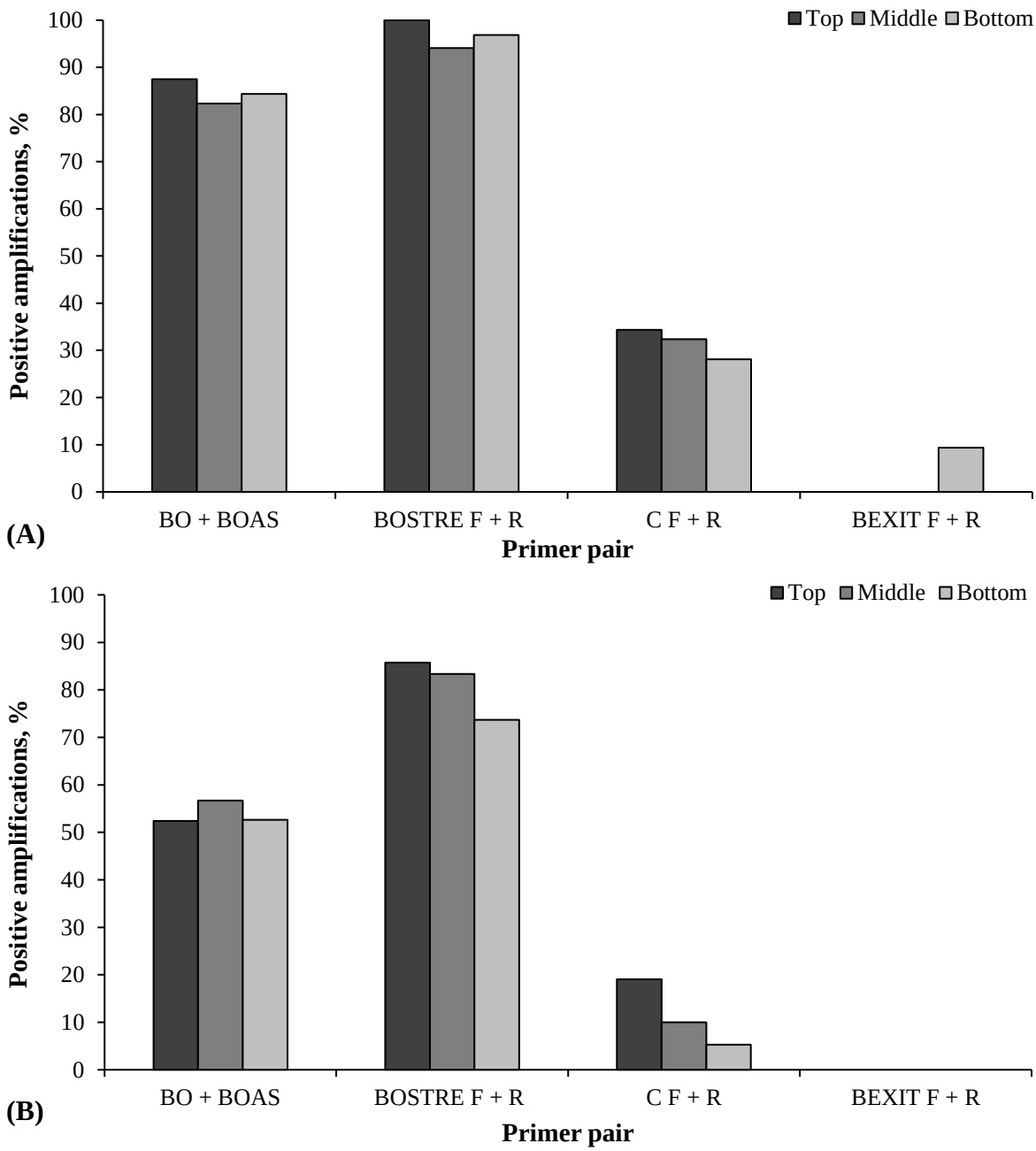


Figure 5.6. Presence of *Bonamia ostreae* (BO+BOAS, BOSTRE-F+BOSTRE-R and C_F+C_R) and *Bonamia exitiosa* (BEXIT-F+BEXIT-R) DNA within *Ostrea edulis* populations in (A) the top (n = 32), middle (n = 34) and bottom (n = 32) micro-reef units of broodstock cages in 2017, and (B) the top (n = 21), middle (n = 30) and bottom (n = 19) micro-reef units of broodstock 2018.

5.3.1.4. Saxon Wharf

Of the 17 broodstock oysters tested in 2017, 100 % provided positive amplifications for *O. edulis* DNA. Presence of *B. ostreae* DNA varied with primer sets used with BO + BOAS providing 82.4 %, BOSTRE 88.2 % and $C_F + C_R$ 17.6 %. *Bonamia exitiosa* DNA was not detected using the BEXIT primer pair (Fig. 5.7A).

Of the 10 broodstock oysters tested in 2018, 100 % provided positive amplifications for *O. edulis* DNA. Again, presence of *B. ostreae* DNA varied with primer sets used with BO + BOAS providing 40 %, BOSTRE 60 % and $C_F + C_R$ 10 %. These values represent a 7.6 - 42.4 % decrease from that in the previous year. *Bonamia exitiosa* DNA was not detected using the BEXIT primer pair (Fig. 5.7A).

Two individuals were observed brooding larvae during the 2017 season, the brood of which was analysed for pathogen presence. Neither *B. ostreae* nor *B. exitiosa* DNA was detected in this adult individual using any of the primer pairs (Fig. 5.7B). The larvae present within the pallial cavity of this individual were found to contain the DNA of *B. ostreae* but the presence of *B. exitiosa* DNA was not detected. An additional brood from this location was analysed and no evidence of *B. ostreae* or *B. exitiosa* DNA was detected.

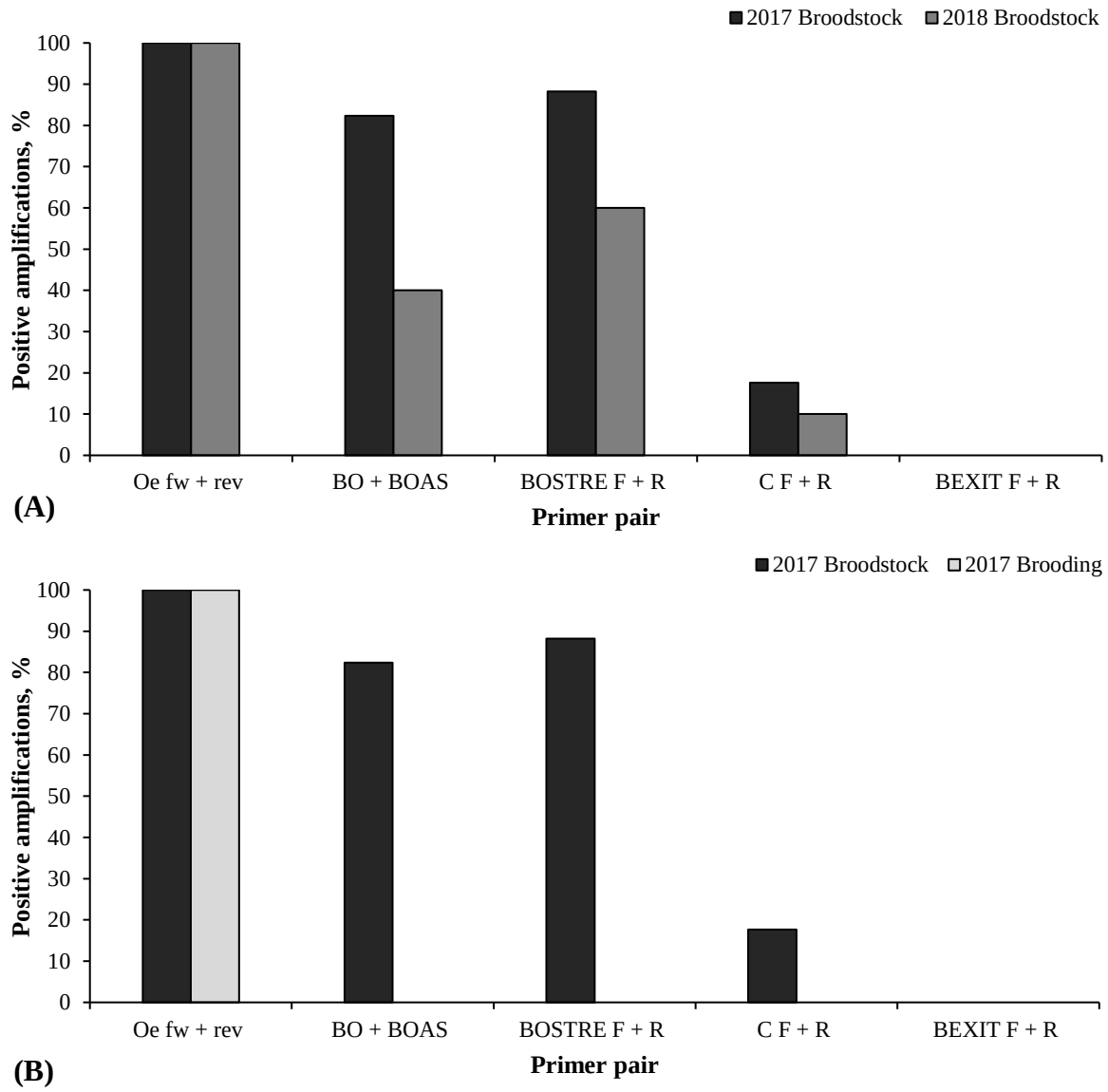


Figure 5.7. Percentage of oysters tested that provided positive results for *Ostrea edulis* DNA using the Oe fw+rev primer pair, *Bonamia ostreae* DNA using the BO+BOAS, BOSTRE-F+BOSTRE-R and C_F+C_R primer pairs, and *Bonamia exitiosa* DNA using the BEXIT-F+BEXIT-R primer pair in (A) 2017 broodstock cages (n = 17) and 2018 broodstock cages (n = 10), and (B) 2017 broodstock cages and 2017 brooding adults (n = 1) within those cages, from Saxon Wharf.

5.3.1.5. Port Hamble

Of the 17 broodstock oysters tested in 2017, 100 % provided positive amplifications for *O. edulis* DNA. Presence of *B. ostreae* DNA varied with primer sets used with BO + BOAS providing 94.1 %, BOSTRE 100.0 % and $C_F + C_R$ 35.3 %. *Bonamia exitiosa* DNA was detected in two oysters (11.8 %) using the BEXIT primer pair (Fig. 5.8A). The contig sequences formed from the *B. exitiosa* PCR-amplification products showed 99.18 and 100 % identity with *B. exitiosa* clone Oeq41A4 from North Carolina (JF831588.1).

Of the 12 broodstock oysters tested in 2018, 100 % provided positive amplifications for *O. edulis* DNA. Again, presence of *B. ostreae* DNA varied with primer sets used with BO + BOAS providing 41.7 %, BOSTRE 58.3 % and $C_F + C_R$ 25.0 %. These values represent a 10.3 - 52.4 % decrease from that in the previous year. *Bonamia exitiosa* DNA was not detected using the BEXIT primer pair (Fig. 5.8A).

Four oysters were observed brooding larvae during the 2017 season, three of these were analysed for pathogen presence. *Bonamia ostreae* DNA was detected in one individual (33.3 %) using the BO + BOAS primer pair and two oysters using the BOSTRE primer pair (66.6 %). The $C_F + C_R$ primer pair did not provide any positive amplifications. *Bonamia exitiosa* DNA was not detected in these oysters using the BEXIT primer pair (Fig. 5.8B).

The larvae present within the pallial cavity of one of these individuals was found to contain the DNA of *B. ostreae* using the BO + BOAS primer pair and two individuals using the BOSTRE primer pair.

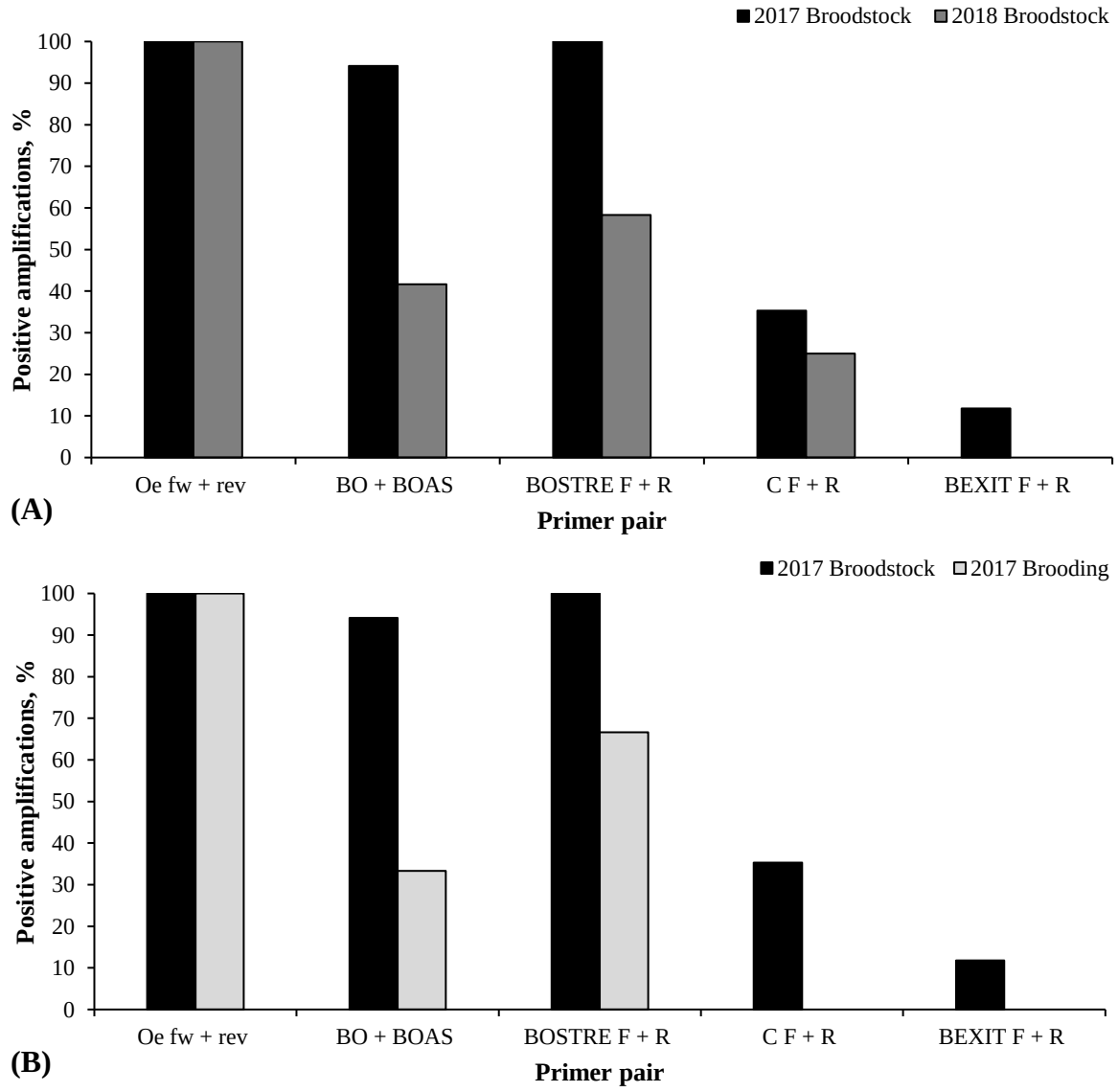


Figure 5.8. Percentage of oysters tested that provided positive results for *Ostrea edulis* DNA using the Oe fw+rev primer pair, *Bonamia ostreae* DNA using the BO+BOAS, BOSTRE-F+BOSTRE-R and C_F+C_R primer pairs, and *Bonamia exitiosa* DNA using the BEXIT-F+BEXIT-R primer pair in (A) 2017 broodstock cages (n = 17) and 2018 broodstock cages (n = 12), and (B) 2017 broodstock cages and 2017 brooding adults (n = 3) within those cages, from Port Hamble Marina.

5.3.1.6. Hamble Point

Of the 17 broodstock oysters tested in 2017, 100 % provided positive amplifications for *O. edulis* DNA. Presence of *B. ostreae* DNA varied with primer sets used with BO + BOAS providing 64.7 %, BOSTRE 100 % and $C_F + C_R$ 35.3 %. *Bonamia exitiosa* DNA was not detected using the BEXIT primer pair (Fig. 5.9A).

Three oysters were observed brooding larvae during the 2017 season, *B. ostreae* DNA was detected in one individual (33.3 %) using the BO + BOAS primer pair and two oysters using the BOSTRE primer pair (66.6 %). The $C_F + C_R$ primer pair did not provide any positive amplifications. *Bonamia exitiosa* DNA was not detected in these oysters using the BEXIT primer pair (Fig. 5.9B). The larvae present within the pallial cavity of two these individuals were found to contain the DNA of *B. ostreae*. An additional brood from this location were analysed and *B. ostreae* and *B. exitiosa* were not present.

Of the 12 broodstock oysters tested in 2018, 100 % provided positive amplifications for *O. edulis* DNA. Again, presence of *B. ostreae* DNA varied with primer sets used with BO + BOAS providing 66.7 %, BOSTRE 91.7 % and $C_F + C_R$ 8.3 %. These values represent an 8.3 - 27 % decrease from that in the previous year. *Bonamia exitiosa* DNA was not detected using the BEXIT primer pair.

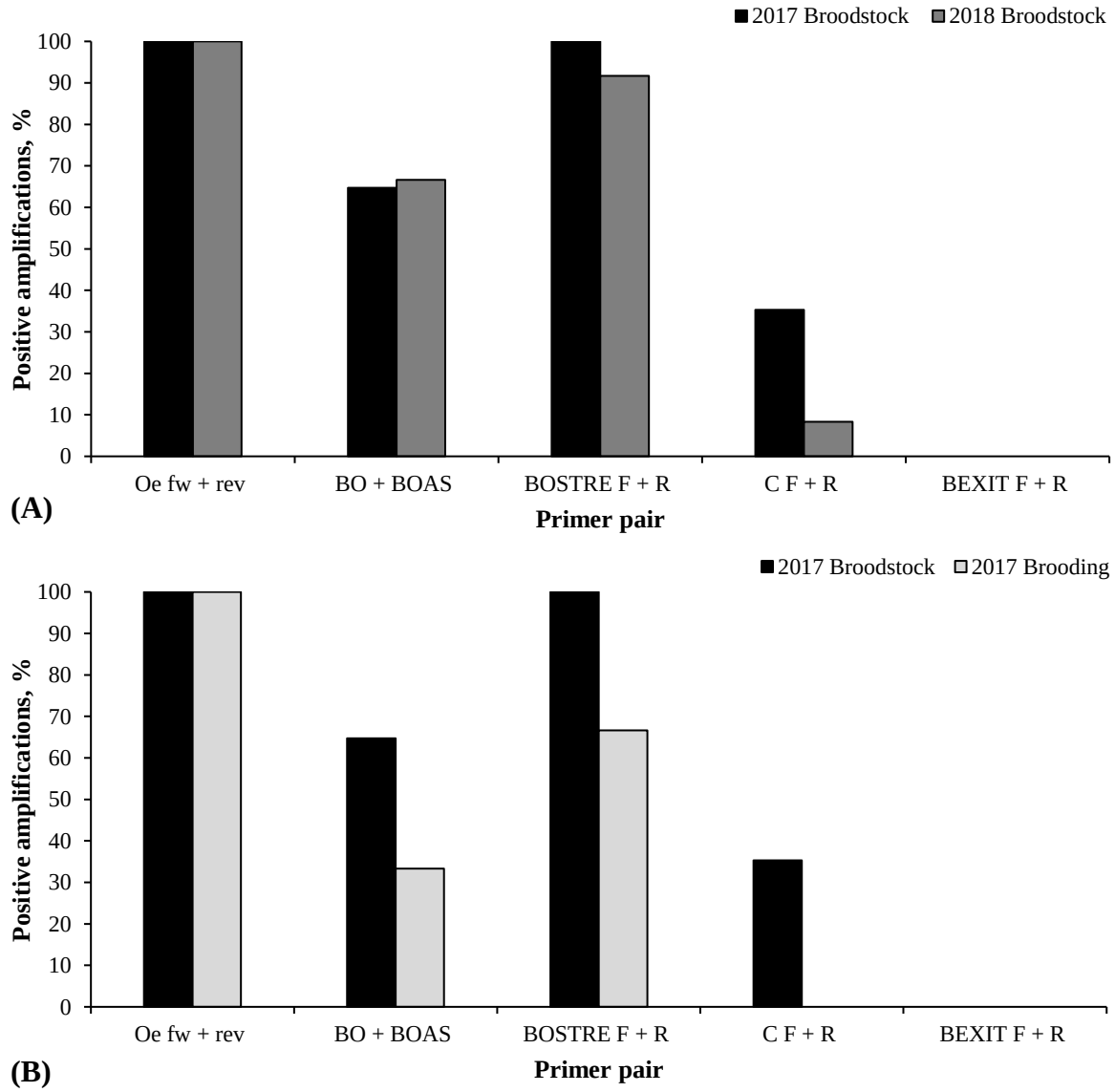


Figure 5.9. Percentage of oysters tested that provided positive results for *Ostrea edulis* DNA using the Oe fw+rev primer pair, *Bonamia ostreae* DNA using the BO+BOAS, BOSTRE-F+BOSTRE-R and C_F+C_R primer pairs, and *Bonamia exitiosa* DNA using the BEXIT-F+BEXIT-R primer pair in (A) 2017 broodstock cages (n = 17) and 2018 broodstock cages (n = 12), and (B) 2017 broodstock cages and 2017 brooding adults (n = 3) within those cages, from Hamble Point Marina.

5.3.1.7. Portsmouth Harbour

The original 2015 fishery population outside the mouth of Portsmouth Harbour provided 100 % positive amplifications for *O. edulis* DNA. Presence of *B. ostreae* DNA varied with primer sets used with BO + BOAS providing 18.8 %, BOSTRE 25 % and C_F + C_R 0 %. *Bonamia exitiosa* DNA was detected in one individual (2.1 %) using the BEXIT primer pair (Fig. 5.10 and Fig. 5.11A). The contig sequence of the *B. exitiosa* PCR-amplification product showed 99.59 % identity with a *B. exitiosa* clone OST79B6 from Tunisia (JF831718.1).

Broodstock cages tested at the end of the 2016 trial, originally derived from the fisher population, provided 100 % positive amplifications for *O. edulis* DNA. Presence of *B. ostreae* DNA varied with primer sets used with BO + BOAS providing 23.8 %, BOSTRE 33.3 % and C_F + C_R 0 %. These values represent a 0 - 8.3 % increase from the previous year. *Bonamia exitiosa* DNA was not detected using the BEXIT primer pair (Fig. 5.11A).

Of the 17 broodstock oysters tested in 2017, 100.0 % provided positive amplifications for *O. edulis* DNA. Presence of *B. ostreae* DNA varied with primer sets used with BO + BOAS providing 88.2 %, BOSTRE 94.1 % and C_F + C_R 17.6 %. *Bonamia exitiosa* DNA was detected in one individual (6.3 %) using the BEXIT primer pair (Fig. 5.11B). The sequence of the *B. exitiosa* PCR-amplification product showed 100 % identity with *B. exitiosa* clone GR176_9 from Australia (JF831683.1).

Of the 12 broodstock oysters tested in 2018, 100 % provided positive amplifications for *O. edulis* DNA. Again, presence of *B. ostreae* DNA varied with primer sets used with BO + BOAS providing 58.3 %, BOSTRE 83.3 % and C_F + C_R 0 %. These values represent a 10.8 - 29.9 % decrease from that in the previous year. *Bonamia exitiosa* DNA was not detected using the BEXIT primer pair (Fig. 5.11B).

Two oysters were observed brooding larvae during the 2017 season, *B. ostreae* DNA was detected in one individual (50 %) using the BO + BOAS primer pair and both oysters using the BOSTRE primer pair. The C_F + C_R primer pair did not provide any positive amplifications. *Bonamia exitiosa* DNA was not detected in these oysters using the BEXIT primer pair (Fig. 5. 11B).

The larvae present within the pallial cavity of both of these individuals were found to contain DNA of *B. ostreae*, *B. exitiosa* DNA was not present. An additional two broods were analysed from this location, both of which contained the DNA of *B. ostreae* but not *B. exitiosa*.

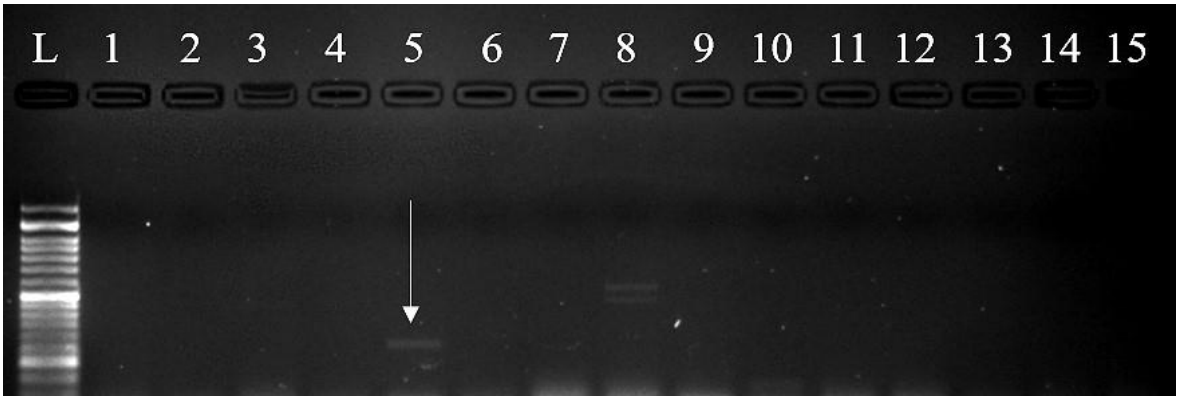


Figure 5.10. *Bonamia exitiosa* DNA present within the gill tissue of *Ostrea edulis*. Detection of *B. exitiosa* in an oyster collected from Portsmouth Harbour, Solent, in November 2015 using the BEXIT-F+BEXIT-R primer set (Lane 5, 246 bp). Lane L represents the 100bp ladder (PCR Biosystems Ltd). The sequence of the PCR-amplification product showed 99.59 % homology with *B. exitiosa* from Tunisia (JF831718.1).

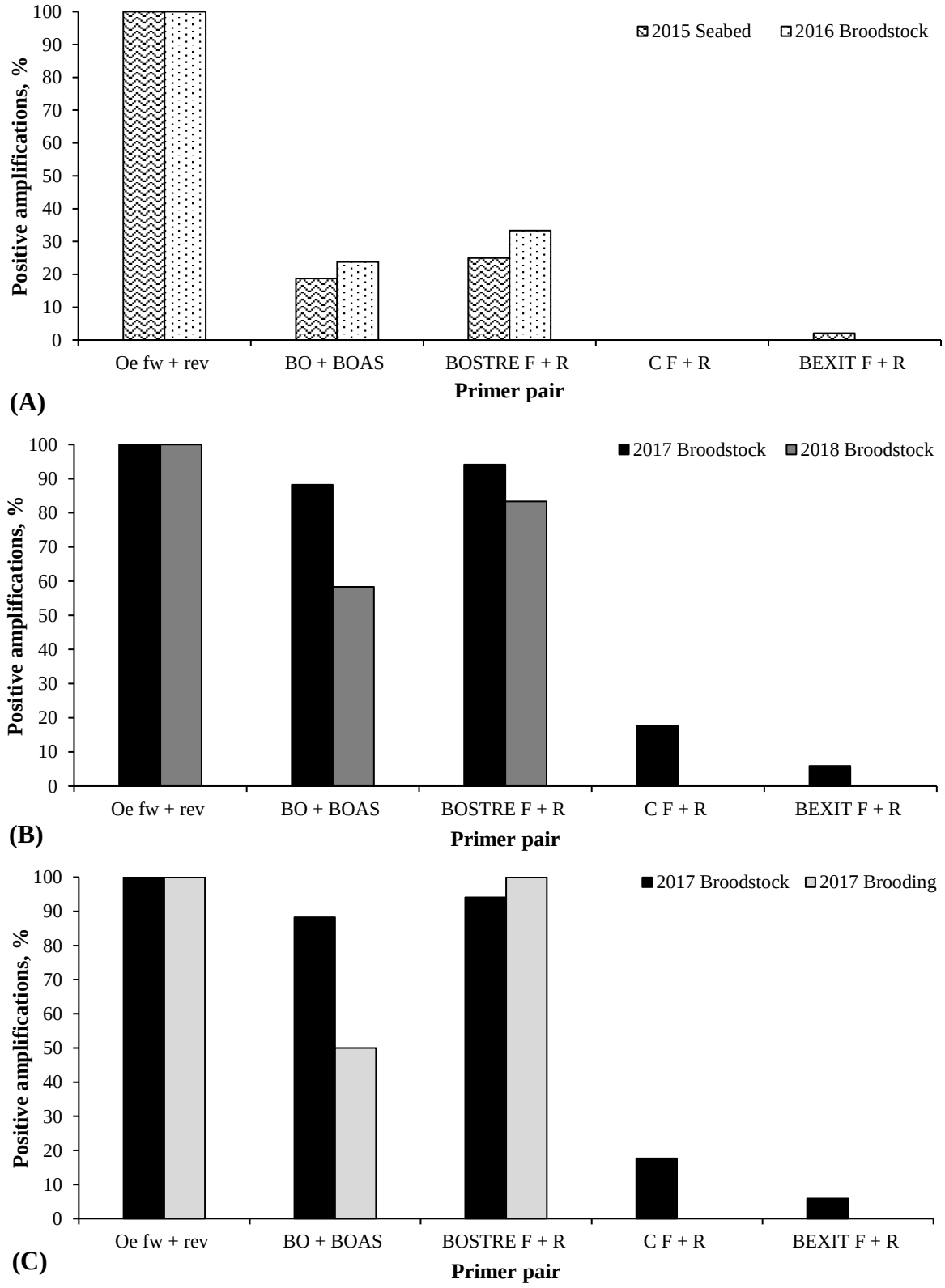


Figure 5.11. Percentage of oysters tested that provided positive results for *Ostrea edulis* DNA using the Oe fw+rev primer pair, *Bonamia ostreae* DNA using the BO+BOAS, BOSTRE-F+BOSTRE-R and C_F+C_R primer pairs, and *Bonamia exitiosa* DNA using the BEXIT-F+BEXIT-R primer pair in (A) 2015 seabed oysters (n = 48) and 2016 broodstock oysters (n = 42), (B) 2017 broodstock cages (n = 17) and 2018 broodstock cages (n = 12), and (C) 2017 broodstock cages and 2017 brooding adults (n = 2) within those cages, from Portsmouth Harbour.

5.3.1.8. Chichester Harbour 2015 fishery and Langstone Harbour 2016 broodstock

The original fishery population from within Chichester Harbour that were later placed into cages within Langstone Harbour provided 100 % positive amplifications for *O. edulis* DNA. Presence of *B. ostreae* DNA varied with primer sets used with BO + BOAS providing 50 %, BOSTRE 72.9 % and $C_F + C_R$ 18.8 %. *Bonamia exitiosa* DNA was not detected using the BEXIT primer pair (Fig. 5.12).

Broodstock cages tested at the end of the 2016 trial, originally derived from the fisher population, provided 100 % positive amplifications for *O. edulis* DNA. Presence of *B. ostreae* DNA varied with primer sets used with BO + BOAS providing 62.5 %, BOSTRE 81.3 % and $C_F + C_R$ 43.8 %. These values represent an 8.4 - 25 % increase from the previous year. *Bonamia exitiosa* DNA was not detected using the BEXIT primer pair (Fig. 5.12).

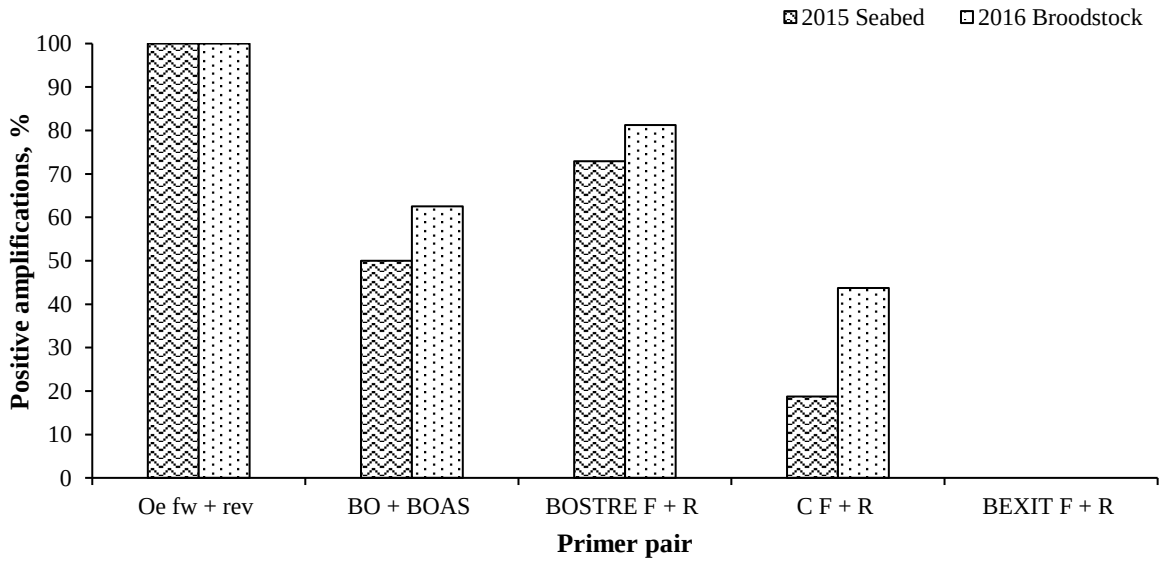


Figure 5.12. Percentage of oysters tested that provided positive results for *Ostrea edulis* DNA using the Oe fw+rev primer pair, *Bonamia ostreae* DNA using the BO+BOAS, BOSTRE-F+BOSTRE-R and C_F+C_R primer pairs, and *Bonamia exitiosa* DNA using the BEXIT-F+BEXIT-R primer pair in the 2015 Chichester Harbour fishery population (n = 48) and 2016 broodstock cages (n = 16) suspended in Langstone Harbour, orignally from the Chichester fishery population.

5.3.1.9. Langstone Harbour

Of the 17 broodstock oysters tested in 2017, 100 % provided positive amplifications for *O. edulis* DNA. Presence of *B. ostreae* DNA varied with primer sets used with BO + BOAS providing 94.1 %, BOSTRE 100 % and C_F + C_R 52.9 %. *Bonamia exitiosa* DNA was not detected using the BEXIT primer pair (Fig. 5.13A).

Five oysters were observed brooding larvae during the 2017 season, *B. ostreae* was detected in two of these (40 %) using both the BO + BOAS and BOSTRE primer pairs. The C_F + C_R primer pair did not provide any positive amplifications. *Bonamia exitiosa* DNA was not detected in these oysters using the BEXIT primer pair (Fig. 5.13B). The larvae present within the pallial cavity of four of these individuals were analysed and were all found to contain DNA of *B. ostreae*, one of which provided a faint band indicating the possibility of DNA from *B. exitiosa*. A contig was not formed from the sequences received from sequencing but the forward sequence of the *B. exitiosa* PCR-amplification product showed 98.59 % identity with *B. exitiosa* close L119_3_4 from Argentina (JF831559.1) and the reverse sequence showed 100 % identity with *B. exitiosa* clone Ost79B6 from Tunisia (JF831718.1). Further analysis by CEFAS could not confirm that this was *B. exitiosa*. Four additional broods were analysed and were also all found to contain the DNA of *B. ostreae*, with one of these also containing the DNA of *B. exitiosa*. Again, a contig of the two sequences could not be formed due to poor sequence quality, the forward sequence of the *B. exitiosa* PCR-amplification product showed 77.90 % identity with *B. exitiosa* clone Ost79B5 from Tunisia (JF831717.1). Of the 12 broodstock oysters tested in 2018, 100 % provided positive amplifications for *O. edulis* DNA. Again, presence of *B. ostreae* DNA varied with primer sets used with BO + BOAS providing 58.3 %, BOSTRE 91.70 % and C_F + C_R 16.7 %. These values represent an 8.3 - 36.2 % decrease from that in the previous year. *Bonamia exitiosa* DNA was not detected using the BEXIT primer pair.

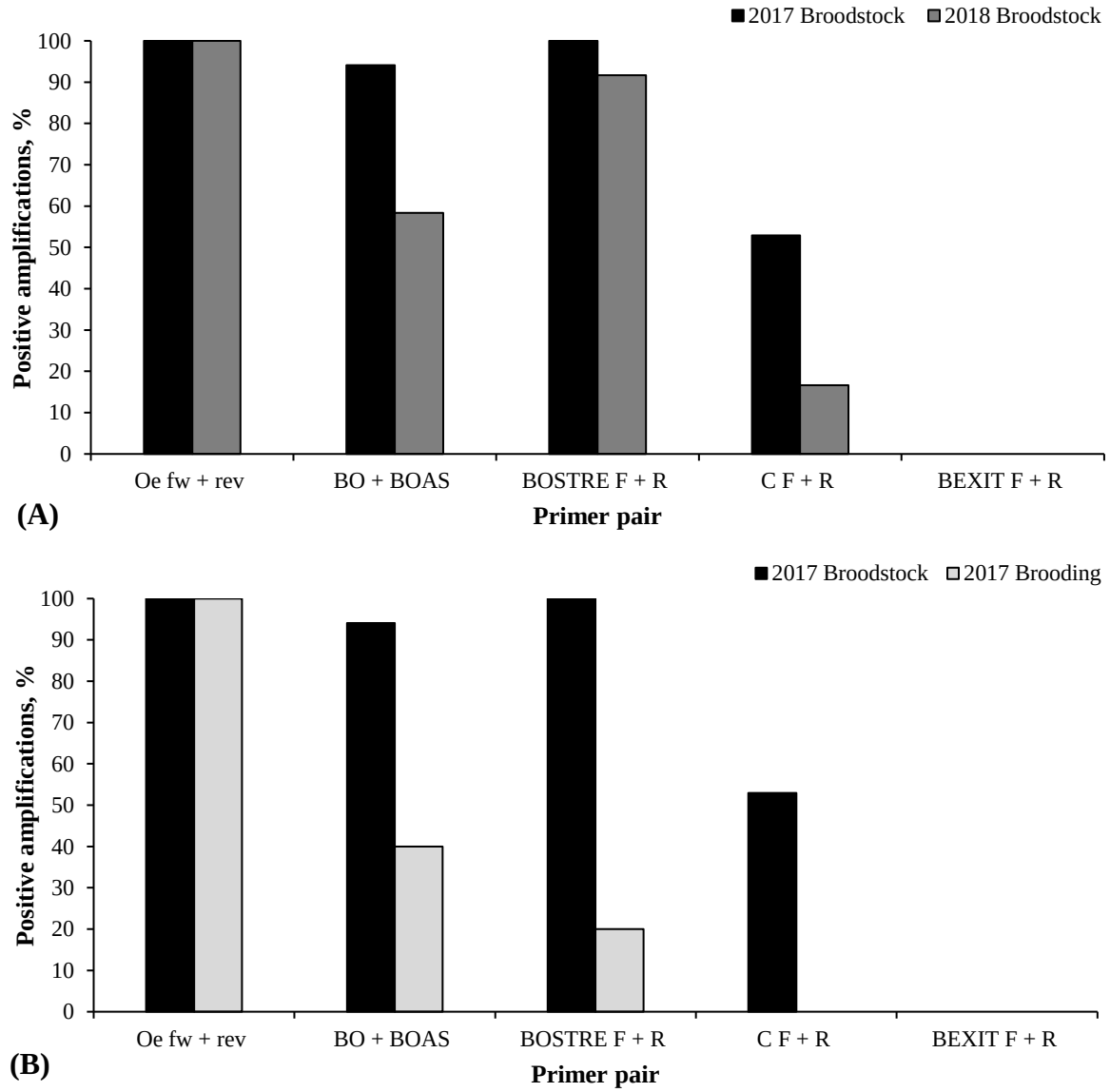


Figure 5.13. Percentage of oysters tested that provided positive results for *Ostrea edulis* DNA using the Oe fw+rev primer pair, *Bonamia ostreae* DNA using the BO+BOAS, BOSTRE-F+BOSTRE-R and C_F+C_R primer pairs, and *Bonamia exitiosa* DNA using the BEXIT-F+BEXIT-R primer pair in (A) 2017 broodstock cages (n = 17) and 2018 broodstock cages (n = 12), and (B) 2017 broodstock cages and 2017 brooding adults (n = 5) within those cages, from Langstone Harbour.

5.3.1.10. Sparkes Marina

Of the 14 broodstock oysters tested in 2017, 13 (92.9 %) provided positive amplifications for *O. edulis* DNA. A single individual (7.1 %), morphologically identified as *Crassostrea gigas*, tested positive for *C. gigas* DNA, this individual tested positive for presence of *B. ostreae* DNA using the BO+ BOAS and BOSTRE primer pairs, no evidence of *B. exitiosa* DNA was detected using the BEXIT primer pair. The sequence of the *C. gigas* PCR-amplification product showed 99.96 % identity with the genome (KJ855245.1). Presence of *B. ostreae* DNA within those that were determined to be *O. edulis* varied with primer sets used with BO + BOAS providing 92.3 %, BOSTRE 100 % and $C_F + C_R$ 30.8 %. *Bonamia exitiosa* DNA was not detected using the BEXIT primer pair (Fig. 5.14A).

Of the 12 broodstock oysters tested in 2018, 100 % provided positive amplifications for *O. edulis* DNA. Again, presence of *B. ostreae* DNA varied with primer sets used with BO + BOAS providing 45.5 %, BOSTRE 63.6 % and $C_F + C_R$ 27.3 %. These values represent a 0 - 34 % decrease from that in the previous year. *Bonamia exitiosa* DNA was not detected using the BEXIT primer pair (Fig. 5.14A).

Eight oysters observed brooding larvae during the 2017 season were analysed, *B. ostreae* DNA was detected in four oysters (50 %) using both the BO + BOAS and BOSTRE primer pairs. The BO + BOAS primers detected *B. ostreae* in one individual that the BOSTRE primer pair did not, and vice versa with the BOSTRE detecting *B. ostreae* in one individual that the BO + BOAS did not. The $C_F + C_R$ primer pair did not provide any positive amplifications. *Bonamia exitiosa* DNA was not detected in these adult oysters using the BEXIT primer pair (Fig. 5.14B). Of the larvae present within the pallial cavity of these adults, four contained DNA of *B. ostreae*, with two of these also containing DNA of *B. exitiosa*. The contig sequence of the *B. exitiosa* PCR-amplification product from one individual showed 100 % identity with *B. exitiosa* clone Oeq41A4 from North Carolina

(JF831588.1). A contig could not be formed from the sequences obtained for the other larval brood due to the low quality of the forward sequence, however, the reverse sequence showed 100 % identity with *Bonamia* sp. Clone B from New South Wales, Australia (JF831683.1). A third brood also contained *B. exitiosa* DNA but not *B. ostreae*. Again no contig could be formed but the forward sequence of the *B. exitiosa* PCR-amplification product from this individual showed 97.77 % identity with *B. exitiosa* isolate H8 from New Zealand (KY680634). An additional brood from 2017 was analysed without the adult comparison and found to contain both *B. ostreae* and *B. exitiosa* DNA. The forward sequence of the *B. exitiosa* PCR-amplification product from this individual showed 93.70 % identity with *B. exitiosa* clone Ocon6B_4 from California (JF831733.1). The reverse sequence of the *B. exitiosa* PCR-amplification product from this individual showed 98.40 % identity with *B. exitiosa* clone Ost79B6 from Tunisia (JF831718.1).

Four additional broods were analysed from the 2018 broodstock cages, three of these individuals were found to contain the DNA of *B. ostreae*, with one also containing the DNA of *B. exitiosa*. The contig sequence of the *B. exitiosa* PCR-amplification product showed 100 % identity with *B. exitiosa* clone Oeq41A4 from North Carolina (JF831588.1). The adults brooding the larvae were not analysed as the larvae were extracted when they were anesthetised to avoid sacrificial sampling.

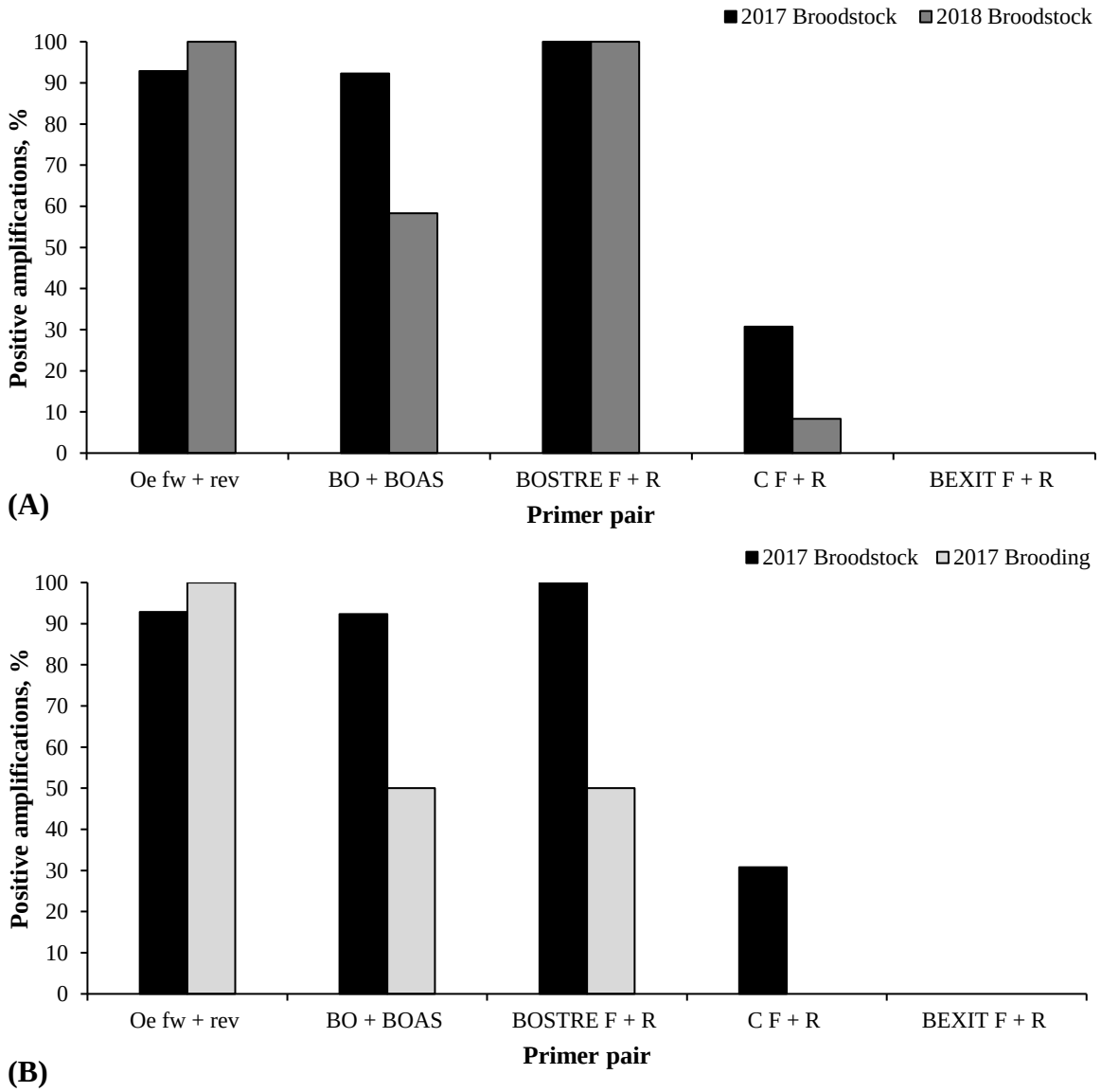


Figure 5.14. Percentage of oysters tested that provided positive results for *Ostrea edulis* DNA using the Oe fw+rev primer pair, *Bonamia ostreae* DNA using the BO+BOAS, BOSTRE-F+BOSTRE-R and C_F+C_R primer pairs, and *Bonamia exitiosa* DNA using the BEXIT-F+BEXIT-R primer pair in (A) 2017 broodstock cages (n = 14) and 2018 broodstock cages (n = 12), and (B) 2017 broodstock cages and 2017 brooding adults (n = 8) within those cages, from Sparkes Marina.

Further analysis is required to confirm the identity of the potential *B. exitiosa* samples from Langstone Harbour and Sparkes Marina larval samples from 2017 that did not provide a contig due to the low sequence quality or non-identity of the forward or reverse sequences. The identity and sequences obtained are highlighted in Table 5.5 with the locations and number of samples analysed shown in Table 5.6. A comparison of the six locations used during the 2017 and 2018 trail is summarised in Figures 5.15 and 5.16.

Table 5.5. Details of samples that tested positive for *Bonamia exitiosa* from sequence analysis, gained using the BEXIT-F+BEXIT-R primer pair, against GenBank entries. Samples where a contig could not be formed and that require further work to confirm identity are grouped with respective border lines.

Sample group	Location	Species	Sequence	Identity %	Geographic region	Host species	Clone	GenBank accession
2015 Seabed	Portsmouth	*B. exitiosa*	Contig	99.59	Tunisia	*Ostrea stentina*	Ost79B6	JF831718
2017 Broodstock	Port Hamble	*B. exitiosa*	Contig	99.18	North Carolina	*Ostrea stentina*	Oeq41A4	JF831588
2017 Broodstock	Port Hamble	*B. exitiosa*	Contig	100.00	North Carolina	*Ostrea stentina*	Oeq41A4	JF831588
2017 Broodstock	Portsmouth	*B. exitiosa*	Contig	100.00	Australia	*Saccostrea glomerata*	GR176_9	JF831683
2017 Larvae	Chichester	*B. exitiosa*	Contig	100.00	North Carolina	*Ostrea stentina*	Oeq41A4	JF831588
2018 Larvae	Chichester	*B. exitiosa*	Contig	100.00	North Carolina	*Ostrea stentina*	Oeq41A4	JF831588
2017 Larvae	Langstone	*B. exitiosa*	F	98.59	Argentina	*Ostrea stentina*	L119_3_4	JF831559
2017 Larvae	Langstone	*B. exitiosa*	R	100.00	Tunisia	*Ostrea stentina*	Ost79B6	JF831718
2017 Larvae	Chichester	*B. exitiosa*	F	97.77	New Zealand	*Ostrea chilensis*	Isolate H8	KY680634
2017 Larvae	Chichester	N/A	R	N/A	N/A	N/A	N/A	N/A
2017 Larvae	Chichester	N/A	F	N/A	N/A	N/A	N/A	N/A
2017 Larvae	Chichester	*Bonamia sp.*	R	100.00	Australia (NSW)	*Saccostrea glomerata*	B	JX977122
2017 Larvae	Chichester	*B. exitiosa*	F	93.70	California	*Ostrea conchaphila*	Ocon6B_4	JF831733
2017 Larvae	Chichester	*B. exitiosa*	R	98.40	Tunisia	*Ostrea stentina*	Ost79B6	JF831718

Table 5.6. Summary of *Ostrea edulis* sample populations, sample type, number of oysters from each location and population sampled. Bold numbers in parentheses indicate the number of positive *Bonamia exitiosa* samples from the respective sample set obtained using the sequence contig, numbers not in bold indicate those samples where possible identification requires further analysis.

		Number of oysters per location								
Sampling year	**Sample type**	**Portsmouth Harbour**	**Chichester Harbour**	**SW**	**PH**	**HP**	**BA**	**UP**	**SP**	**Total**
2015 Seabed populations	Gill	48 **(1)**	48							96
2016 Broodstock cages	Gill						42	16		58
2017 Broodstock cages	Gill + Heart			17	17 **(2)**	17	17 **(1)**	17	14	99
2017 Brooding individuals (within cages)	Gill + Heart			1	3	3	2	5	8	22
2017 Larvae	Larvae			2	4	4	4	8 **(1)**	9 **(1,3)**	31
2018 Broodstock cages	Gill + Heart			10	12	12	12	12	12	70
2018 Larvae	Larvae								4 **(1)**	4

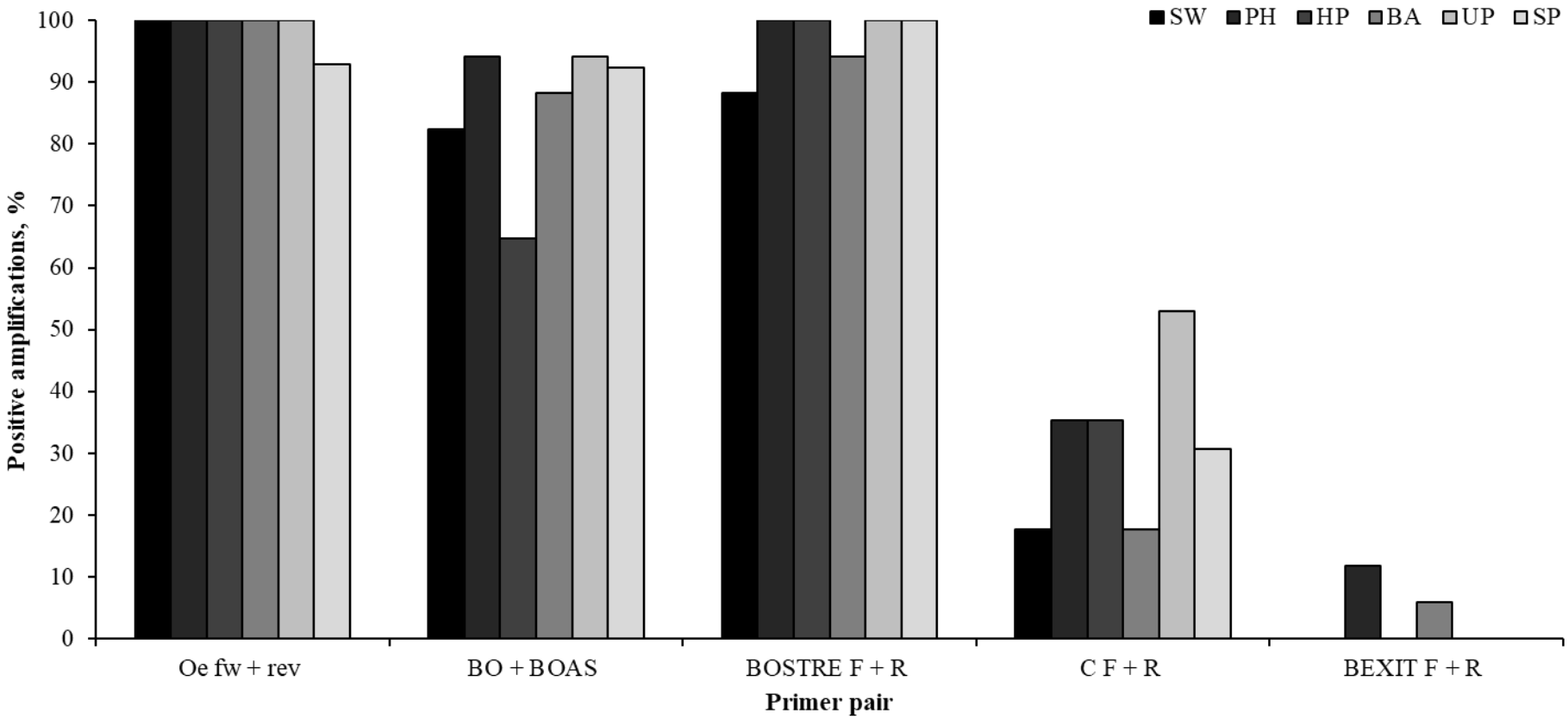


Figure 5.15. Percentage of oysters tested that provided positive results for *Ostrea edulis* DNA using the Oe fw+rev primer pair, *Bonamia ostreae* DNA using the BO+BOAS, BOSTRE-F+BOSTRE-R and C_F+C_R primer pairs, and *Bonamia exitiosa* DNA using the BEXIT-F+BEXIT-R primer pair in 2017 broodstock cages from Saxon Wharf (SW), Port Hamble Marina (PH), Hamble Point Marina (HP), Portsmouth Harbour (BA), Langstone Harbour (UP) and Sparkes Marina (SP).

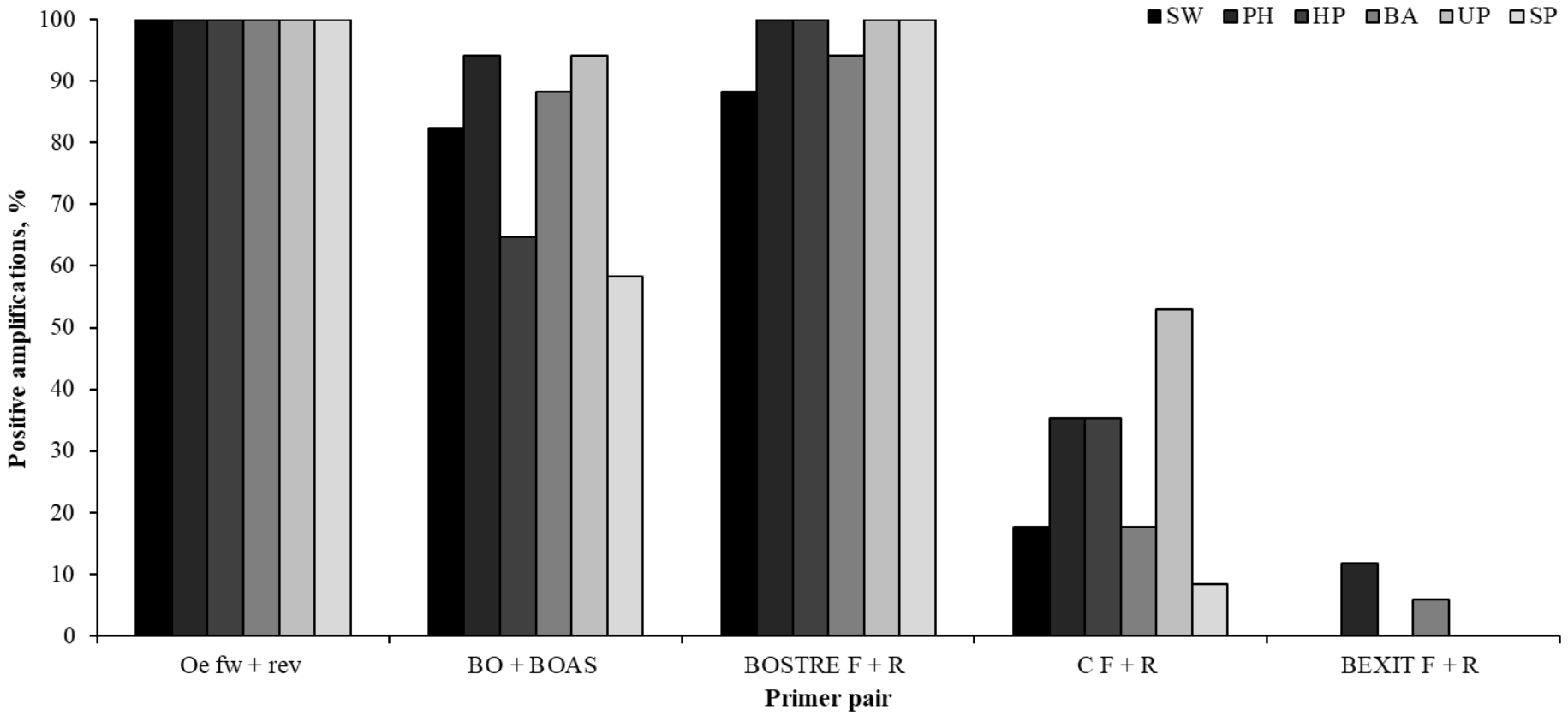


Figure 5.16. Percentage of oysters tested that provided positive results for *Ostrea edulis* DNA using the Oe fw+rev primer pair, *Bonamia ostreae* DNA using the BO+BOAS, BOSTRE-F+BOSTRE-R and C_F+C_R primer pairs, and *Bonamia exitiosa* DNA using the BEXIT-F+BEXIT-R primer pair in 2018 broodstock cages from Saxon Wharf (SW), Port Hamble Marina (PH), Hamble Point Marina (HP), Portsmouth Harbour (BA), Langstone Harbour (UP) and Sparkes Marina (SP).

5.3.2. Phylogenetic analysis

Detection of the positive *B. exitiosa* samples indicate that the short fragment amplified by the BEXIT-F + BEXIT-R primer pair is more sensitive than the BO + BOAS, but further analysis revealed that the short sequence provided does not enable enough phylogenetic variation to be detected in order to establish evolutionary relationships (Table 5.7). However, from the limited variation observed there do appear to have been separate introduction events, shown by the similarity of Solent samples to samples from various geographic origins (Figures 5.17 and 5.18).

Table 5.7. Estimates of Evolutionary Divergence between Sequences. The number of base differences per site from between sequences are shown. This analysis involved 18 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 589 positions in the final dataset. Evolutionary analyses were conducted in MEGA X v. 6 (Kumar *et al.*, 2018).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	B. exitiosa in *O. edulis* (UK 2015 Adult1)																	
1	*B. exitiosa* in *O. edulis* (UK; 2017 Larvae)	0.0041																
2	*B. exitiosa* in *O. edulis* (UK; 2017 Adult2)	0.0041	0.0000															
3	*B. exitiosa* in *O. edulis* (UK; 2017 Adult3)	0.0082	0.0041	0.0041														
4	*B. exitiosa* in *O. edulis* (UK; 2017 Adult4)	0.0041	0.0000	0.0000	0.0041													
5	*B. exitiosa* in *O. edulis* (UK; 2018 Larvae)	0.0041	0.0000	0.0000	0.0041	0.0000												
6	*B. exitiosa* in *C. ariakensis* (FL, USA; JF712867)	0.0163	0.0123	0.0123	0.0082	0.0122	0.0123											
7	*B. exitiosa* in *O. angasi* (AUS; JF831680)	0.0122	0.0082	0.0082	0.0041	0.0082	0.0082	0.0052										
8	*B. exitiosa* in *O. chilensis* (NZL; JF831655)	0.0163	0.0123	0.0123	0.0082	0.0121	0.0123	0.0052	0.0052									
9	*B. exitiosa* in *O. conchaphila* (CA, USA; JF831723)	0.0122	0.0082	0.0082	0.0041	0.0081	0.0082	0.0034	0.0034	0.0017								
10	*B. exitiosa* in *O. puelchana* (ARG; JF831633)	0.0163	0.0123	0.0123	0.0082	0.0122	0.0123	0.0035	0.0052	0.0052	0.0035							
11	*B. exitiosa* in *O. stentina* (ARG; JF831573)	0.0122	0.0082	0.0082	0.0041	0.0082	0.0082	0.0034	0.0052	0.0035	0.0034	0.0035						
12	*B. exitiosa* in *O. stentina* (NC, USA; JF831588)	0.0123	0.0082	0.0082	0.0041	0.0082	0.0082	0.0034	0.0034	0.0017	0.0000	0.0035	0.0034					
13	*B. exitiosa* in *O. stentina* (NZL; JF831661)	0.0163	0.0123	0.0123	0.0082	0.0122	0.0123	0.0034	0.0052	0.0052	0.0034	0.0035	0.0034	0.0034				
14	*B. exitiosa* in *O. stentina* (SC, USA; JF831599)	0.0122	0.0082	0.0082	0.0041	0.0082	0.0082	0.0034	0.0052	0.0052	0.0034	0.0035	0.0034	0.0034	0.0034			
15	*B. exitiosa* in *O. stentina* (TUN; GU356032)	0.0122	0.0082	0.0082	0.0041	0.0082	0.0082	0.0017	0.0034	0.0035	0.0017	0.0017	0.0017	0.0017	0.0017	0.0017		
16	*B. exitiosa* in *S. glomerata* (AUS; JF831683)	0.0122	0.0082	0.0082	0.0041	0.0081	0.0082	0.0069	0.0069	0.0052	0.0034	0.0070	0.0052	0.0035	0.0069	0.0069	0.0052	
17	*B. ostreae* in *O. edulis* (ME, USA; AF162087)	0.7622	0.7663	0.7663	0.7663	0.7647	0.7663	0.7525	0.7505	0.7525	0.7565	0.7546	0.7546	0.7576	0.7546	0.7561	0.7546	0.7566

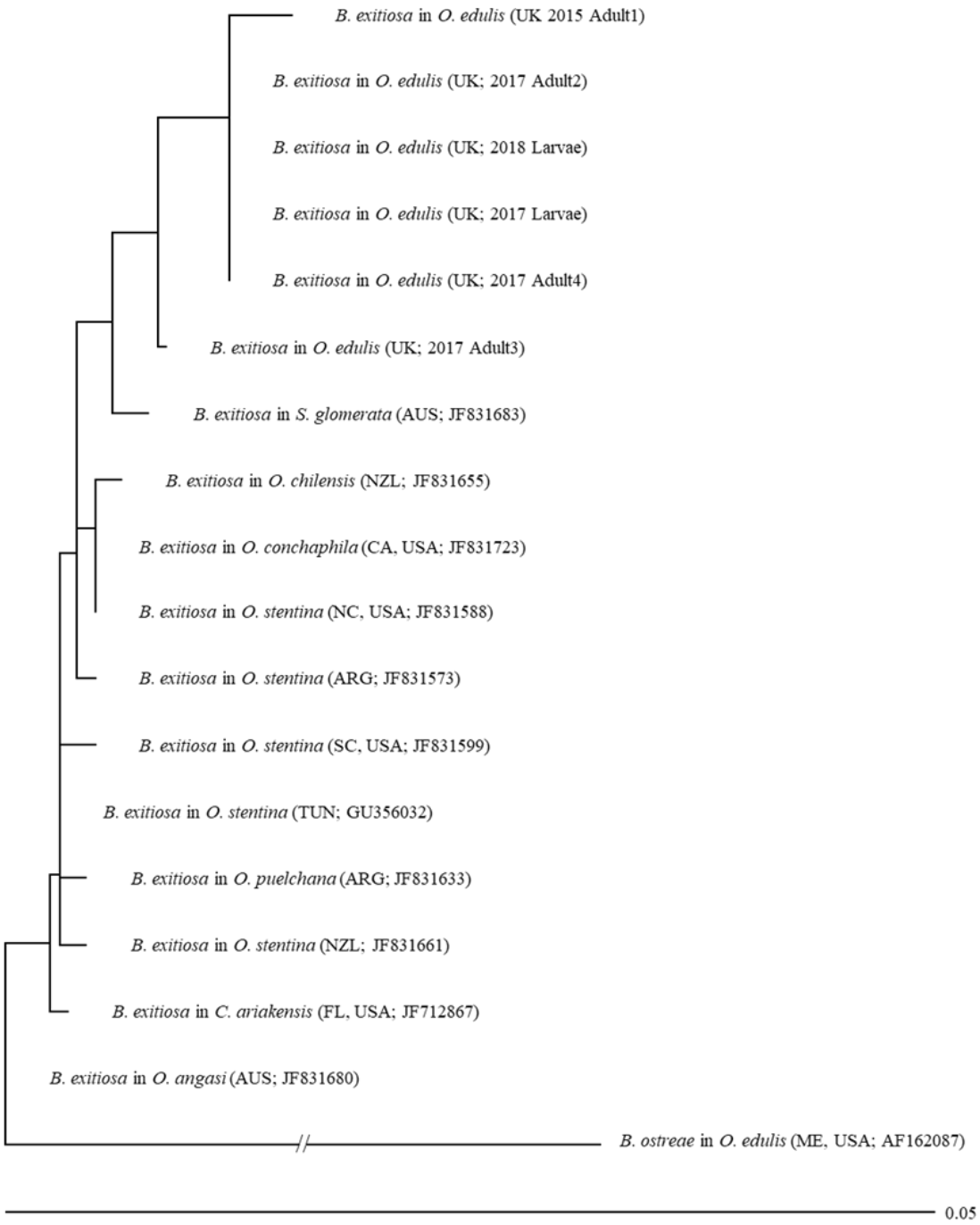


Figure 5.17. *Bonamia* spp. small subunit (SSU) 18S rRNA gene and internal transcribed spacer 1 (ITS1) Neighbor-Joining tree. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The outgroup of *Bonamia ostreae* is not shown to scale, indicated by //, due to the excessive evolutionary distance. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. This analysis involved 18 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 589 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018). AUS: Australia; NZL: New Zealand; CA: California; NC: North Carolina; SC: South Carolina; FL: Florida; ARG: Argentina; TUN: Tunisia.

Figure 5.18. *Bonamia* spp. small subunit (SSU) 18S rRNA gene and internal transcribed spacer 1 (ITS1) Maximum Likelihood tree. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model (Tamura, 1992). The tree with the highest log likelihood (-1605.94) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The outgroup of *Bonamia ostreae* is not shown to scale, indicated by //, due to the excessive evolutionary distance. This analysis involved 18 nucleotide sequences. There was a total of 589 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018). AUS: Australia; NZL: New Zealand; CA: California; NC: North Carolina; SC: South Carolina; FL: Florida; ARG: Argentina; TUN: Tunisia.

5.4. Discussion

This chapter summaries the presence of pathogenic species within the oyster populations sampled and there is the emphasis that, although the presence of *B. ostreae* and *B. exitiosa* DNA has been detected and verified with the use of DNA sequencing, it is not clear if these are infective individual pathogens causing a detrimental impact to the host oysters or, if they are simply present within the tissue. Hence, the possibility that the oysters were not diseased or the parasitic cells of *B. exitiosa* were not viable cannot be completely ruled out (Burreson, 2008). Further histological analysis would have allowed for any indication of this, unfortunately this could not be conducted within the time frame of this study.

The current diagnostic techniques, for the detection of *B. ostreae*, are outlined by the World Organisation for Animal Health (OIE) (OIE, 2019). Within the molecular techniques section there is the recommendation for the use of the BO + BOAS (Cochennec *et al*., 2000), C_F + C_R (Carnegie *et al*., 2000) or BOOF03 + BOOSR03 (Engelsma *et al*., 2010) primer pairs. The efficacy of the three primer pairs BO + BOAS, C_F + C_R and BOSTRE (Ramilo *et al*., 2013) was assessed in this study and showed that results varied substantially, with the C_F + C_R primer pair detecting far fewer incidences of positive samples than the BO + BOAS and BOSTRE primer pairs. It is clear that the use of the C_F + C_R primer pair is not efficient and should no longer be recommended for diagnostics, the first author of the publication is in agreement with this conclusion (R. Carnegie, pers. comm.). Across all samples, the BOSTRE primer pair detected the DNA of *B. ostreae* within more oysters that the BO + BOAS primer pair, indicating an increased sensitivity. It is recommended that this evidence be confirmed with further histological analysis.

Once confirmed the OIE molecular methodology for *B. ostreae* detection should be updated, with general and specific revisions needed to the whole chapter surrounding

infection with *B. ostreae*. In particular, the use of the BOSTRE primer pair should be advised before the use of BO + BOAS primer pair. The comment in the disadvantages of PCR technique 'Does not allow pathogen species determination (needs to be combined with RFLP + sequencing for species characterisation)' should also be amended, as the species specific primers, BOSTRE and BEXIT designed by Ramilo *et al*. (2013) now allow for this determination.

The OIE also outlines the current diagnostic analysis techniques for the detection of *B. exitiosa* (OIE, 2019). Again, the molecular methodology for infection with *B. exitiosa* should be updated, with the recommendation for the use of the BO + BOAS and C_F + C_R primer pairs to detect the DNA of *B. exitiosa* unlikely to provide accurate results of infection, none of the samples confirmed to be positive in this study, obtained with the use of the BEXIT primer pair, were detected by either BO + BOAS or C_F + C_R primer pairs. False negatives were also detected by Ramilo *et al*. (2013) and Batista *et al*. (2016) further supporting the case for the removal of the BO + BOAS or C_F + C_R primer pairs from the OIE recommendations.

Results from all years of this study support the hypothesis that increasing oyster density increases pathogen prevalence within the populations. This hypothesis provides the basis to the OSPAR and CEFAS density recommendations for *O. edulis* beds of five and ten oysters / m^2, respectively (OSPAR, 2009; Laing *et al.*, 2005). The presence from within 2015 and 2016 populations indicate that the number of oysters with *B. ostreae* DNA present within them increased when translocated from the original fishery population into suspended broodstock cages. Initially the proportion of the population from Portsmouth Harbour fishery with *B. ostreae* DNA present was lower than that from Chichester Harbour. When using the BO + BOAS and C_F + C_R primer pairs the increase in pathogen presence was greater in those oysters translocated from the Chichester Harbour fishery into Langstone Harbour than for

those translocated from the Portsmouth Harbour fishery area to the Camber Dock, however, the difference using the apparently more sensitive BOSTRE primer pair was negligible. This could be an indication that the increase in disease prevalence within a translocated full-density population increases to a similar extent regardless of the initial prevalence of the source population. Therefore, selecting source populations with a low prevalence may prolong the infection of unaffected oysters and provide those individuals with additional time to reproduce.

The results from 2016, 2017 and 2018 again indicate that *B. ostreae* increased in prevalence substantially over a twelve month period when translocated into cages, from being present within a relatively low proportion (4.1%) of the initial stocking population, to a high proportion (> 88.2 %) of the broodstock cage populations distributed across the Solent in 2017. The percentage of the population found to have *B. ostreae* DNA within them then decreased the following year at five of the six sample locations. The stocking density within the marinas as a whole could have influenced this increase in prevalence, with the populations in 2017 comprised of approximately 1,400 individuals / marina and by the sampling period in 2018 were reduced to approximately 360 oysters / marina. Although distributed vertically, when considering the horizontal two-dimensional space occupied by the cages the initial stocking density would equate to approximately 280 / m^2 (1,400 oysters distributed across five hatches ~ 1 m^2 in size) and the final stocking density would equate to approximately 120 / m^2 (360 oysters distributed across three hatches ~ 1 m^2 in size). These densities vastly exceed the recommended densities for disease prevention as well as the natural capacity for the species as it would be physically impossible for reefs of adult oysters to reach these abundances on the seabed.

Another potential explanation for the decreased prevalence in 2018 could be that the full-density and relatively stressful conditions increased selection for individuals with the

ability to resist infection by the pathogen. This argument could be supported by the survival and normal, or relatively high, condition indices of these surviving individuals at all locations, despite incidents of large mortalities within the rest of the populations (see Chapter 3).

The presence of *B. ostreae* DNA within larvae brooded by adults during 2017 and 2018 supports the current understanding that *B. ostreae* can indeed be present within the larvae of *O. edulis* (Arzul *et al.*, 2011; Flannery *et al.*, 2016). Both previous studies analysed samples using the BO + BOAS primer pair so are likely to have under reported the number of adults and larvae infected. Incidences of infected adults and larvae, uninfected adults with infected larvae, infected adults with uninfected larvae and, uninfected adults and larvae were found using both the BO + BOAS and BOSTRE primer pairs. More larval broods were found to be infected than brooding adults. These findings further support the suggestion that larvae are likely to contribute to the spread of *B. ostreae*.

The presence and establishment of *B. ostreae* across much of Europe means it will be impossible to eradicate the pathogen completely and that biosecurity measures in place will only help to prevent and prolong the further spread into naïve populations. There is evidence that *B. ostreae* can persist in areas even in areas where *O. edulis* has been completely removed, infecting any introduced individuals (van Banning, 1998). Lynch *et al*. (2007) indicate that this could be due to the ability of *B. ostreae* to infect multiple macroinvertebrate hosts. All eight of which, observed in that study, are present across the Solent area and much of Europe. The presence of *B. ostreae* in the 2007 study was not limited to benthic species, 19 grouped zooplankton species also yielded positive results adding to the information that the copepod *Paracartia grani* acts as an intermediate host for *Marteilia refringens*, another serious oyster pathogen (Audemard *et al.*, 2002).

The detection of *B. ostreae* DNA within the tissue of *Crassostrea gigas* in this study supports the findings of Lynch *et al*. (2010), however, unlike some specimens analysed in the previous study, *B. exitiosa* DNA was not detected in the individual Pacific oyster sampled in this study. This detection should prompt further investigation into the role that Pacific oysters play in the transmission and dispersal of *B. ostreae* and *B. exitiosa* within the Solent system. Recently, after particularly warm spring and summer periods, populations of *C. gigas* across the Solent have expanded and increased in abundance in much of the available intertidal, and shallow subtidal habitat (pers. obs.), an ecological niche left available due to the continued exploitation of *O. edulis*. Another non-native species that has dramatically increased in abundance and distribution across the Solent is the American slipper limpet *Crepidula fornicata* (Chapter 2, also in Helmer *et al*., 2019). Again, little or no information is available on the susceptibility of this now dominant invasive mollusc to *B. ostreae*, or *B. exitiosa*, in the subtidal habitat and should be investigated further.

This parasitic establishment within the environments that *O. edulis* inhabits, or once inhabited, emphasises the need for the continuation of selective breeding programs for *O. edulis* which will be vital in determining and developing resistance to *B. ostreae*. Many trials have been conducted and others are ongoing, experiencing varying levels of success in comparative growth, mortality and immune gene expression (Martin *et al*., 1993; Baud *et al*., 1997; Naciri-Graven *et al*. 1999; Culloty *et al*., 2001, 2004; Lallias *et al*., 2009, Martín-Gómez *et al*., 2012; Lynch *et al*., 2014; Morga *et al*., 2017).

The relatively full-density *O. edulis* broodstock cage systems trialled in this study could provide a mechanism for selective breeding of individuals that survive, grow and reproduce in the somewhat hostile environment at the surface of the water column. Not only is this likely to select for more disease tolerant, or potentially resistant, individuals but also

those that are likely to be more adapt to future predicted environmental conditions, such as increased temperature (IPCC, 2018).

The additional discovery of *B. exitiosa* in this study describes the second detection of this pathogen in the UK and the first within the Solent. The earliest sample populations collected and preserved in 2015, and analysed in 2019, represent individuals that we from a natural seabed population. The detection in oysters collected prior to the broodstock cage trials conducted in this study indicates that the species was likely introduced into the area during one of the many translocations of European flat oysters that have taken place across Europe (Wolff and Reise, 2002; Bromley *et al*., 2016), whether that be reported or unreported. This may also be the case for movements or natural spread of other commercially important, and susceptible, species such as *C. gigas*. Another explanation, supported by the findings of this study, could be that the pathogen was transported via the larval distribution of *O. edulis* or other susceptible species from previously infected populations (Abollo *et al.*, 2008; Narcisi *et al.*, 2010; Arzul *et al.*, 2010; Arzul *et al*., 2011; Carrasco *et al.*, 2010; Longshaw *et al.*, 2013; Batista *et al.*, 2016).

The presence of *B. exitiosa* DNA within larvae brooded within adults during 2017 and 2018 in this study provides additional positive detections to that found within the larval brood of an individual *O. edulis* on the French Atlantic coast (Arzul *et al*., 2011). The samples from France were analysed using the BO + BOAS primer pair so are likely to have under reported the number of adults and larvae infected with *B. exitiosa* as none of the broodstock or larvae shown to be infected using the BEXIT primer pair provided positive amplifications with the BO + BOAS primer pair. Only larval broods within the adults were found to be infected using the BEXIT primer pair in this study, none of their respective parent oysters were shown to be infected but other non-brooding adult oysters were, as previously described. Positive results were confirmed from three of the six sample locations

use to house broodstock oysters, with the distribution of these locations spanning the area from River Hamble to the eastern Harbours of the Solent. Concurrent infection of *B. ostreae* and *B. exitiosa* was detected in all but one of the samples using both primers for *B. ostreae* and the single primer for *B. exitiosa*. These findings suggest that, along with infective particles (Hine, 1991a, b; Diggles and Hine, 2002), larvae are likely to contribute to the spread of *B. exitiosa*.

It is not yet clear how *B. exitiosa* will interact with populations of *O. edulis* in the UK due to the apparently recent introduction and limited number of incidents within relatively few oysters. The potential adaptations, towards disease tolerance, of some *O. edulis* populations to the pathogen-host relationship with *B. ostreae* may limit or prevent mass mortality events due to *B. exitiosa*. The presence of these pathogens within the larvae stage of the *O. edulis* lifecycle may be contributing to a hidden mortality cost during development, however, this could result in those no succumbing to infection due to tolerance or resistance, or those uninfected surviving to maturity and could be the reason so few adults tested positive. It is yet to be determine how the level of mortality due to ontogenetic-specific pathogenic infection differs from natural mortality during this time in the life history. This could provide an opportunity for concentrated disease resistance selection, with many of the broods containing > 1,000,000 larvae subjecting them to disease exposures would likely be more effective than exposing mature individuals for the selection process. Once these individuals reach an appropriate age and size the genes associated with resistance (Martín-Gómez *et al.*, 2012) could be targeted and breeding programs could be established from such populations.

Another possibility is that interspecific competition between the two pathogens is occurring, with *B. ostreae* excluding or outcompeting *B. exitiosa* in many cases, this level of interaction is yet to be examined in these species.

Due to the reduced prevalence of *B. ostreae* and complete absence of *B. exitiosa* within brooding adults, in comparison with the whole population. It could be inferred that those individuals not infected are physiologically more robust than those expending additional energy to mediate and fight the presence of the pathogen or pathogens (Culloty *et al.*, 2007) and therefore have additional metabolic reserves that can be assigned to gonadal growth and / or sex change to produce larvae. The increased prevalence with the larval broods in comparison to the brooding adults could be as a result of external horizontal transmission when the parent oyster is uninfected, alternatively there is the possibility that it could be associated with a transport mechanism of pathogen, with the microcells transferring from adult haemocytes and extracellular into the larvae prior to their release from the parent oyster to assist with dispersal into other locations.

Chapter 6

General Discussion and Recommendations

6.1. Completion of study objectives

As detailed in the general introduction, this study aimed to address and assess the following:

- current densities and distribution of *Ostrea edulis* in relation to *Crepidula fornicata*;
- density and distribution of *O. edulis* and *C. fornicata* over a substantial time period;
- multiple years of fishery data to determine any trends in population demographics;
- efficacy of suspended broodstock cages with regards to survival and condition;
- timing and extent of reproduction in these adult populations;
- alterations to the reported skewed sex ratio;
- success of larval recruitment in areas where broodstock cages were introduced;
- larval abundances in relation to *Crassostrea gigas* and *C. fornicata*;
- pathogen prevalence within seabed and broodstock cage populations (including larval broods);
- environmental limits for broodstock cage systems and;
- recommendations for suspended cages as broodstock systems in restoration projects

The success of the study with regards to these objectives is summarised in the following sections, with conclusions, recommendations and identification of knowledge gaps that have arisen from the results. As previously mentioned, it was not possible to address all of the objectives due to time and technical restraints, but the core principles of the study provide valuable and substantial insights into the restoration of *O. edulis* in the Solent.

6.2. Current status of *Ostrea edulis* in the Solent

The evidence for the collapse of the fishery within the Solent is now unequivocal and the necessity for active management is more apparent than ever before. This global crisis has been driven by the removal of broodstock and the associated habitat destruction (Rothschild *et al.*, 1994; Beck *et al.*, 2011) that has exacerbated issues associated with other detrimental factors. The data collected during this study shows that even over a recent time period, the previous 20 years, an already depleted baseline has shifted further from that of historic abundances. The true baseline of the Solent is currently unknown and should be investigated using paleoecological, archaeological, anecdotal, biological and biogeochemical evidence (Pauly, 1995; Cranfield *et al.* 1999; Jackson *et al.*, 2001; Rick and Lockwood, 2013). Evidence that can be used to infer the habitat does, however, exists for the Solent region and should be further investigated to obtain a detailed understanding of the habitat historically inhabited by *O. edulis* (Winder, 1992 in Campbell, 2010). Without this perception of previous abundances and habitat, conservation or restoration targets for *O. edulis* across Europe could be under ambitious, as seen with other marine programmes in the North Sea and elsewhere (Plumeridge and Roberts, 2017). Restoration practitioners across Europe are, however, in the fortunate situation where the appropriate management mechanisms for success are available from decades of global experience in other geographic regions ([USA] Brumbaugh *et al.*, 2006; [Global] Beck *et al.*, 2009, 2011; [Australia] Gillies *et al.*, 2015, 2017; [USA] zu Ermgassen *et al.*, 2016) and have allowed the sharing of best practice to be an integral component of projects from their inception (Pogoda *et al.*, 2019).

The general consensus for ocean optimism stories enables engagement of policy makers, multiple other stakeholders and the general public (McAfee and Connell, 2019; McAfee *et al.*, 2019b); the story of *O. edulis* epitomises this concept of ocean optimism and

the multiple benefits of restoration can be easily redirected to the audience in question. Balancing the optimistic and pessimistic viewpoints surrounding the current status of *O. edulis* restoration will be key to long term sustained success (McAfee and Connell, 2019).

The subsequent proliferation of *C. fornicata* within the eastern Harbours of the Solent, since the introduction from its natural range along the Atlantic seaboard of the US (Collin, 2001), poses one of the most imminent threats to the recovery the of the native flat oyster in the subtidal habitats once occupied by *O. edulis* and is a prime example of a situation that provides strong optimistic and pessimistic opinions. *Crepidula fornicata* is extremely versatile and resilient, having been found at subtidal depths of 60 m in high current velocities off the coast of the Isle of Wight (Hinz *et al.*, 2011), in temperatures ranging from near freezing to 30°C (Rayment, 2001; Walne, 1956), salinities from 18 ‰ to fully saline and in clean to eutrophic environments (Rayment, 2001). With these broad tolerances and little or no predation pressure from native benthic species, such as *Carcinus maenas* or *Asterias rubens* (Thieltges *et al.*, 2004) and native fish species, such as dab, only thought to feed on exposed individuals (Fitzgerald, 2007), there are few factors preventing their ecological dominance.

This phenomenon has not been limited to the Solent, with high densities of *C. fornicata* having been recorded since their establishment in Essex (Walne, 1956, Truro (Fitzgerald, 2007), Wales (Bohn *et al.*, 2012), France (Chauvaud, 1998; de Montaudouin and Sauriau, 1999), the Netherlands (Nienhuis, 1992) as well as other areas in Europe from the Norwegian coast down to the Mediterranean Sea (Blanchard, 1997; McNeill *et al.*, 2010; Hinz *et al.*, 2011). The presence of live *C. fornicata* also has direct and indirect ecological and economic implications. Despite not actually killing commercially important species of oyster, mussel or scallop, they do compete for sustenance and substrata, thus limiting or reducing populations of these native species (Fresard and Boncoeur, 2004; Fresard and Boncoeur,

2005; Fresard *et al.*, 2005). Economically this is detrimental, as within the UK, *C. fornicata* is not readily consumed in the restaurant trade meaning that their removal from the benthos, sorting from desirable species, removal off of desirable species, disposal, reduction of catch quality, loss of fishing area and loss of finfish habitat (Le Pape *et al.*, 2004) all add financial burdens to those working the area.

Despite de Montaudouin and Sauriau (1999) showing increases in macrozoobenthic diversity, this was in direct comparison to areas of bare mud or sand where only one of five areas having a significant increase. The presence of *C. fornicata* also poses a threat to vulnerable ecosystems such as maerl beds (Grall and Hall-Spencer, 2003). However, the impact that *O. edulis* can provide to benthic diversity and abundance is likely to be more profound and provides a basis for optimism surrounding the species restoration.

The live organisms pose the largest threat to *O. edulis* numbers, but dead *C. fornicata* shell accounted for over 92 % of the total settlement material used by the live chains in this study, suggesting that harrowing of beds to break up the chains and expose fresh shell material for oyster settlement is actually likely be counterproductive in facilitating exponential settlement of juvenile slipper limpets (Clark, 2008; Cook *et al.*, 2015) whilst limiting the settlement of *O. edulis* (Bromley *et al.*, 2016b). Recent evidence has suggested that *C. fornicata* is not well adapted to sediment burial and cannot survive at depths of ≥ 6 cm (Powell-Jennings and Callaway, 2018). The potential to utilise dredge material, that is currently an issue for coastal management (Marmin *et al.*, 2014; Callaway, 2016), or to capture *C. fornicata* in dredge material, presents opportunities to smother or remove *C. fornicata* beds before depositing a foundation of coarse rocks and cultch at the appropriate time to facilitate and encourage settlement of *O. edulis*. Appropriate precautions would need to be taken to ensure the prevention of further spread of an invasive non-native species (INNS) as per the legislation (http://www.legislation.gov.uk/ukpga/1981/69/section/14).

Both the presence of *C. fornicata* and the limited supply of suitable substrata would need to be addressed simultaneously to enable restoration of native oysters. If, as a result, *O. edulis* populations reached sizeable densities and a widespread distribution then their ability to readily consume the larvae of *C. fornicata* (Pechenik *et al.*, 2004) would undoubtedly assist with prevention of further proliferation of this pest species. It is therefore recommended that the possibilities detailed by Fitzgerald (2007) be further explored within the Solent.

6.3. The efficacy of suspended broodstock cages for larval supply

The primary objective of this study was to determine the efficacy of novel broodstock cages as a source of larvae, providing recruitment opportunities in strategically located marinas. The results demonstrated that the concept of this style of aquaculture is feasible, but that technical and logistical processes involved require refinement, in order to improve the sustainability of such practices. The most encouraging results were that a substantial proportion of the adult population displayed brooding activity, which would provide sufficient quantities of larvae to enable an appropriately located, deployed and managed substratum to facilitate substantial recruitment. The timing and extent of this larval production, obtained during the initial year long trial, also provides reliable information that can be utilised to inform the decision making process and allow for the deployment of suitable settlement substrata or spat collectors at the optimum temporal period for the formation of the appropriate biofilm prior to settlement and metamorphosis (Walne, 1974). The timing of larval release could also be assessed with forecasted weather patterns that can either select for retention or dispersal of larvae, allowing the appropriate decision to be made in order to minimise energetic costs associated with larval settlement. Downward propulsion of *Crassostrea virginica* larvae has been shown to be promoted in turbulent conditions

associated with coastal environments (Fuchs *et al.*, 2013, 2015), thus rougher weather conditions would encourage such behaviour and if this were to be the case deployment of settlement substrata in close proximity to broodstock populations would be recommended. Alternatively, weather predictions could indicate calmer conditions allowing for the dispersal of larvae over a greater distance in a less turbulent environment under the same tidal regime if this downward propulsion behaviour was postponed. If this were the case, then the deployment of settlement substrata in the areas predicted by larval dispersal models that are at a greater distance from the broodstock would be suggested. By predetermining locations for deployment of substrata under varying weather conditions it would be expected that the likelihood of successful recruitment could be improved and would reduce the effects of sporadic reproduction.

The impact of stocking density also became apparent with no significant difference in the quantity of adults brooding, but those within the lower density producing significantly more larvae than those housed in the higher densities. Within all brooding adults there was a strong correlation with whole wet weight and larval count, as well as this maximum shell length and depth were also correlated with larval count within the brood. This has implications for the current legislative approach to the minimum landing size of the Solent fishery and an alteration to the measuring device is recommended. These amendments involve a transition from a 70 mm circular ring to a rectangle with internal measurements of 30 mm in depth and 75 - 80 mm in length, this minimum landing size would not be unreasonable as an increase from 75 to 80 mm (circular measurement) was implemented in Lough Foyle for the 2010/11 season (Institute of Fisheries Management, 2012) and has been in place since (C. Bromley, pers. comm.). By implementing such measures, along with the continued closure of sanctuary areas free of bottom towed fishing pressing, the broodstock would be allowed to mature and reach a substantial size and age whereby their brood

capacity, in conjunction with multiple years of reproductive activity, would be likely to provide the recurrent quantities of larvae required to form self-sustaining reefs of *O. edulis*.

One concern with the cage design used in this study is the relatively high cumulative mortality within the broodstock populations over the two-year trial. However, a variety of adaptations can be made to mitigate against seasonal mortality during certain months and to reduce negative effects due to cage design. An example of such modifications is the manner in which individual oysters are housed within the cages, removing the internal micro-reef structures will allow for the addition of removable shelving so that the weight of the oysters and associated fouling at the top of the cage does not impact the structural integrity of the areas towards the bottom of the cage. These changes would reduce the issues surrounding increased mortality at the bottom of the cages due to an inability to feed sufficiently will be removed. This would mean that the quantity of oysters housed in a single cage unit would be reduced but would allow for a greater amount of public engagement if sponsorship and monitoring schemes were developed.

It is also recommended that the cages be lowered so that they are held on, or in close proximity to, the seabed during the warmer summer months from June or July, to August or September. This change in cage management would prevent the cages from being suspended in at the surface of water column, which is most influenced by the external parameters, and would relieve brooding oysters of stressors that likely increased mortality in this study. Furthermore, the likelihood of *O. edulis* larval settlement would increase if the substrata provided by mature individuals was closer to the natural settlement environment. The environmental parameters should be monitored prior to selection of marinas in order to prevent excessive mortality in unsuitable locations. Environments where that salinity is influenced by freshwater inputs and that are subject to stratification should be avoided. Contemporary approaches such as oyster gardening, whereby oyster spat on shell are on-

grown in suspended cages (Brumbaugh and Coen, 2009; Rossi-Snook *et al.*, 2010; Krasny *et al.*, 2014) before deployment to reef structures, should also be strongly considered if seed supply is available from local hatcheries.

6.4. Implications of disease

The impact that *Bonamia ostreae* has had on stocks of *O. edulis* across Europe means that it is one of the major concerns amongst restoration practitioners, hence the inclusion of "Respect *Bonamia*-free areas" within the Berlin Recommendation in an attempt to prolong the spread into uninfected areas (Pogoda *et al.*, 2019). In areas where the pathogen has persisted for a prolonged period eradication is not a viable option and working with the disease to develop resistance (Elston *et al.*, 1987; Martin *et al.*, 1993; Hervio *et al.*, 1995; Boudry *et al.*, 1996; Baud *et al.*, 1997; Naciri-Graven *et al.*, 1998; Culloty *et al.*, 2001, 2004; Lynch *et al.*, 2014; Ballina *et al.*, 2018; Vera *et al.*, 2019) is imperative to the long-term establishment of *O. edulis* populations that have the capacity to tolerate or resist infection.

The findings of this study do indicate that disease prevalence can vary even across small spatial scales and highlight the importance of biosecurity measures, it is therefore suggested that restoration projects employ a policy preventing the movement of oysters once introduced into a specific area. Another recommendation is that all deceased oysters be removed from any restoration aquaculture processes and air-dried for a minimum of six months before the shell is utilised for cultch material. Also, where applicable, the flesh of recently deceased individuals should be disposed of in an appropriate manner (dependant on local legislations) and effort should be taken to avoid this material entering the water column where the oysters are situated.

The results also suggest that a degree of resilience to the presence of *B. ostreae* exists within the Solent population of *O. edulis*. The presence of this pathogen was detected within a relatively high proportion of the various populations throughout the study, yet only a single

event that caused > 50 % mortality occurred within a single population during one month of the 2017 trial was recorded, similarly there was a single mass mortality event that exceeded 90 % at one location at the inception of the 2018 trial. The cause of these mortality events cannot be confirmed and may indeed be a combination of a variety of factors, but it seems unusual that disease-related mortality would be the singular cause and only occur in this way within one or two locations. Furthermore, strong correlations with Chlorophyll-a peaks and freshwater storm input events point to potential drivers of these mass mortalities.

The decrease in prevalence from 2017 to 2018 may also be indicative of survival of individuals that are resistant to the pathogen. A similar occurrence was observed within the brooding population of *O. edulis* during 2017, with a reduction in the percentage of the population observed brooding larvae in comparison with the remaining population. One possible explanation is that those oysters were resistant or uninfected did not experience an energetic burden or reduction in condition often associated with the presence of *B. ostreae* (Culloty, 2007) and were therefore capable of undergoing transitions from male to female state and the ensuing reproductive process. An alternative possibility is that in some cases parasites migrated from the host adult tissue into the larvae during the brooding process in anticipation of the dispersal potential provided by their release. There is also the possibility that the larvae became infected as the parent oyster passes water over them, as *B. ostreae* is able to survive at least one week in the water column (Arzul *et al.*, 2009) and can complete its life cycle without an intermediate host (Hervio *et al.*, 1995), although this alternative route of transmission may also be possible (Lynch *et al.*, 2007).

The survival and associated adaptations of larval broods via selection pressure, along with recent developments in genetic selection mechanisms (Ballina *et al.*, 2018; Vera *et al.*, 2019) could equip aquaculture specialists and restoration practitioners to reduce the impact that *B. ostreae* has on stocks of restored *O. edulis* populations.

6.5. Further recommendations for restoration practices

For Europe-wide restoration of *O. edulis* to successfully occur in an efficient and sustainable manner, the sharing of knowledge, best practice, technology and resources outlined within the Berlin Oyster Recommendation will be essential (Pogoda *et al.*, 2019). By implementing restoration practices in this manner, the scientific and production processes will progress more effectively and will allow for joint funding opportunities that will be integral to large scale activities. For restoration projects to be "successful" goals and quantitative metrics must be defined in order to set a benchmark against which success can be evaluated (Westby *et al.*, 2011; zu Ermgassen *et al.*, 2016).

6.5.1. Site selection and reef design

The initial focus for any restoration project should be based around determining and defining the areas intended for restoration, described by the Berlin Recommendation as "reintroduction", "reinforcement" or "conservation" sites (Pogoda *et al.*, 2019). By utilising the available historical data on locations and abundances of *O. edulis* populations (Barnes, 1973; Key & Davidson, 1981; Utting, 1991; Tubbs, 1999) thorough investigations into the current suitability of the benthic habitat and environmental conditions can be conducted prior the deployment of oysters. Local knowledge, expertise and understanding of fishermen working the area must be utilised and engagement with such stakeholders should be encouraged. However, the advice will likely be in favour of restoring fishery areas and a balance of inclusivity and caution must be achieved, as these areas that once supported abundant fisheries are evidently prime locations for their return, but without the appropriate legislative and physical barriers, could once again become subject to excessive and unsustainable fishing pressure. Locations that contain small or remnant populations and that

are relatively inaccessible, regarding dredging or hand collection activities, offer the appropriate opportunity for protected broodstock locations that, in time, can seed historic fishery locations.

For populations to establish and become self-sustaining, the environmental conditions must fall within the tolerances of *O. edulis*, with the emphasis placed on temperature and salinity. Despite *O. edulis* having a preference for fully saline conditions (Korringa, 1941), stocks do extend into estuarine environments, where salinities of 23 ‰ are tolerated. Hutchinson & Hawkins (1992) showed tolerance of salinities as low as 16 -19 ‰, if the temperature did not exceed 20°C. For example, no oysters survived more than 7 days at 16 psu and 25°C (Hutchinson & Hawkins, 1992); although these combined conditions rarely occurred within the natural biogeographical range of *O. edulis*. It was also noted that at low temperatures, ≤ 10°C, the metabolic rate was minimal, which would increase the prospect of survival in the low salinities associated with storm runoff in the winter months.

The hydrodynamic nature of the system is also of utmost importance and will ultimately influence the dispersal of larvae released by broodstock within the restoration areas. A selection of dispersive and retentive sites are represented by hydrodynamic modelling in the Solent, (Z. Holbrook, pers. comm.) and would provide the optimum combination of retaining populations within protected sanctuary areas whilst also supplying areas that are managed in a sustainable way to allow for small scale extraction.

Appropriate selection of restoration location can have profound impacts on the longevity of reef structure and function, with long-term monitoring essential to the accurate assessment on returns of natural capital investment (Ziegler *et al.*, 2018). Within the Solent the recent introduction of the Bottom Towed Fishing Gear 2016 and Solent Dredge Fishing 2016 byelaws by the Southern Inshore and Fisheries Conservation Authority (IFCA) (Southern IFCA, 2019) provide the legislative protection needed to assist with site selection.

It is highly recommended that if the environmental conditions are suitable, within these areas that are no longer threatened by dredge activities, then this is where restoration efforts should be concentrated. The creation of reef structures should also be conducted in a manner that further deters any dredging activity by any persons that are inclined to neglect the legislative protection. This consideration of structural reef design will also be key to the prosperity of restored populations, with Colden *et al.* (2016) highlighting the influence of *C. virginica* reef orientation, in relation to tidal flow direction, on longevity and oyster performance. Reef height above the sea bed will also be an essential factor incorporated within the design to improve physiological performance (Sawusdee *et al.*, 2015), density, survival and complexity (Colden *et al.*, 2017), and limitation of sediment accumulation (Jordan-Cooley *et al.*, 2011; Colden *et al.*, 2016) to prevent reef habitat loss (Powers *et al.*, 2009; Schulte *et al.*, 2009; Rodriguez *et al.*, 2014; Colden and Lipcius, 2015).

Reef designs should also consider the incorporation of other species to enable and encourage settlement of *O. edulis*. An example shown by the facilitation of *Ostrea angasi* recruitment by *Ecklonia radiata* in southern Australia, where the laminae provide shading, remove sediment and turf algae, increasing the abundance of flat oysters (Shelamoff *et al.*, 2019). With many similar species of kelp native to the UK and Europe it is possible that these relationships also existed on the reefs that once existed and that similar techniques could be employed to facilitate settlement of *O. edulis* on constructed reefs. Opportunities for such trials to occur already present themselves, the Sussex IFCA kelp forest restoration project aims to restore *Laminaria hyperborea*, *Laminaria digitata* and *Saccharina latissima* (Sussex IFCA, 2019) in habitats that are suitable for, and may even still support, *O. edulis*.

Although likely to be controversial, the use of *C. gigas* may also offer the necessary structure required to initiate settlement and proliferation of *O. edulis* as seen in the Dutch part of the North Sea (Christianen *et al.*, 2018). Multiple opportunities are provided by this

now abundant bivalve and acknowledging that their complete eradication is not feasible and could enable the success of *O. edulis* restoration to be improved. Live *C. gigas* could be incorporated into the cultch and sediment deposited to form the reef structure (Walles *et al.*, 2016) to assist with the reduction of *C. fornicata* settlement and as settlement provision for *O. edulis.* Whilst the shell of collected and shucked oysters could be used in a similar manner or be utilised in the production of spat on shell in a hatchery environment. The accessibility of intertidal populations of *O. edulis* contributed to their excessive removal, now, in a similar manner, the accessibility to large quantities of *C. gigas* presents an opportunity for a relatively inexpensive supply of shell material and their collection could provide an opportunity for community-based restoration efforts (Brumbaugh *et al.*, 2006) when collecting substantial quantities of oysters from intertidal areas.

6.5.2. Broodstock or larval sourcing

Once restoration locations and reef designs have been decided, the selection of oysters used to seed the area can be considered. The primary reason which lead to the necessity of restoring *O. edulis* populations across Europe, particularly within the Solent, was the decline in multiple year classes of broodstock that would have historically been self-sustaining (Helmer *et al.*, 2019*).* This lack of individuals capable of producing large quantities of larvae is the primary obstacle facing restoration practitioners. The results of this study demonstrate that environmental conditions and local adaptations, even over a relatively small spatial distribution, can have profound impacts on the reproductive potential of remaining broodstock, up to 400,000 larvae / adult. The results of Utting *et al.* (1991) show no significant difference between the larval production capacities of various *O. edulis* populations across the Solent when sampled *in situ*. In contrast, when translocated, from Langstone Harbour to alternative locations the resulting larval capacity is significantly

reduced with increasing distance from the source location. These findings add further evidence to support the argument of using local stocks for seed supply, as suggested by Bromley *et al.* (2016a) and outlined in the Berlin Recommendation:

"*Translocation between sites of seed oysters or any other size classes from wild beds should be discouraged to avoid increasing the pressure on still existing wild beds and reduce the risk of spreading invasive species and disease. Recommendation: Action should be undertaken to support existing hatcheries, spatting ponds and spat collector techniques and to establish new hatcheries and spatting ponds for the production of robust and genetically diverse* Ostrea edulis *seed. Broodstock sanctuaries should be established and used for local reinforcements."*

In areas where populations of oysters are functionally extinct or completely absent the import of stock should adhere to strict biosecurity measures and broodstock individuals should be housed in conditions that mimic those of the intended deployment location as closely as possible. The most efficient way to achieve this would be to produce larvae within the same system, where possible, or to import stock from the nearest producers geographically. Implications of the transfer of diseases, discussed in 6.3, and invasive species should be at the forefront throughout the decision-making process.

6.5.3. Provisioning of alternative incomes and their potential benefits

By their very nature many restoration projects are initiated with the intention of preventing the removal of oysters from the benthos. This can cause friction and a sense of unease within the fishing community that rely, or previously relied, on the fishery for a source of income. By providing information on the historical habitat and working in collaboration with fishermen, policy makers, the general public and other stakeholders (McAfee *et al.*, 2019a) the development of an aquaculture industry in the area could be

possible. The production of both *O. edulis* and *C. gigas* could be a suitable alternative to encourage the transition from benthic trawling, now illegal in many areas of the Solent, to more sustainable forms of oyster culture with the idea having been reported on in regional news as a potential solution in an attempt to mitigate against nitrate pollution in the area (https://www.bbc.co.uk/news/uk-england-hampshire-49364998). This may also provide the additional benefit of reducing the impacts of disease associated with fishery stress (van Banning, 1991). The potential for social-economic growth in the area would allow for production of *C. gigas* for human consumption and of *O. edulis* for restoration purposes, with the former subsidising the latter. The potential to preserve genetic diversity, as per the final Berlin recommendation (Pogoda *et al.*, 2019), is also presented by cultivating remaining natural stocks in this manner and would allow for introductions of genetic lines to improve resilience within the population.

With increasing coastal development and concurrent human population density, environmental pollution levels will undoubtedly follow this trend. In order to compensate for this, a large responsibility lies with wastewater treatment companies and is likely due to economic reasons, alternative solutions will become a necessity. The ecosystem services provided by marine aquaculture for people and nature, especially of filter feeding species such as oysters, offer an unrivalled solution (Alleway *et al.*, 2018), with the value of marine and coastal habitats globally estimated to be in the region of $50 trillion USD per annum (Costanza *et al.*, 2014). The potential benefits of oyster aquaculture in the Solent extend beyond the obvious provisioning of food supply and improvements to water quality. Numerous migratory and resident wildfowl and wading bird species rely on the habitats within the Solent, hence there are numerous Special Protection Area (SPA) designations (JNCC, 2018). Nichols *et al.* (2019) have shown that the presence of intertidal aquaculture gear encourages foraging behaviours of wading and wildfowl bird species. Modelling of the

improvements in water quality and reduced turbidity in the presence of oysters has predicted that seagrass bed restoration and recovery can be facilitated (Newell and Koch, 2004), these habitats provided by *Zostera* spp. are utilised by wildfowl (Tubbs and Tubbs, 1983).

Oyster grow-out cages, using native species, have also been observed to act as artificial reefs for temperate fish species (Tallman and Forrester, 2007) and support a higher species abundance, richness and diversity than submerged aquatic vegetation and non-vegetated seabed (DeAlteris *et al.*, 2004). In contrast, aquaculture of non-native oyster species has been shown to support lower densities of benthic invertebrates than native seagrass species, but greater densities than unstructured mudflats (Hosack *et al.*, 2006). Aquaculture practices in areas where seagrass beds exist also have a detrimental impact on production, density and size (Tallis *et al.*, 2009), highlighting the importance of specific site selection during the decision-making process of suitable locations for future aquaculture production. The combination of traditional intertidal and floating oyster aquaculture techniques with the novel approach detailed in this study, and that described by Merk *et al.* (in preparation), should be seriously considered and looked upon favourably for the numerous benefits that could be provided within the Solent.

6.6. Conclusion

The low standing stock of *O. edulis*, coupled with a benthos dominated by high densities of *C. fornicata*, the presence of *B. ostreae* and continued fishing pressures are significant barriers to self-sustaining native oyster populations within the Solent. Based on the status of *O. edulis* in the commercially fished areas of the Solent presented here, active management of the seabed is recommended to (1) control the extent and spread of *C. fornicata*, (2) provide suitable settlement substrate for *O. edulis* larval recruitment and (3) establish a protected *O. edulis* broodstock population in all commercially fished Solent Harbours, in agreement with Fariñas-Franco *et al.* (2018). The use of suspended broodstock cages would be recommended as a short to medium-term solution to address larval deficits in recruitment limited areas and as a means of engaging local communities. However, restoration efforts should direct the majority of their efforts into the establishment of protected seabed populations of broodstock and incorporate management strategies that reduce the impact of disease.

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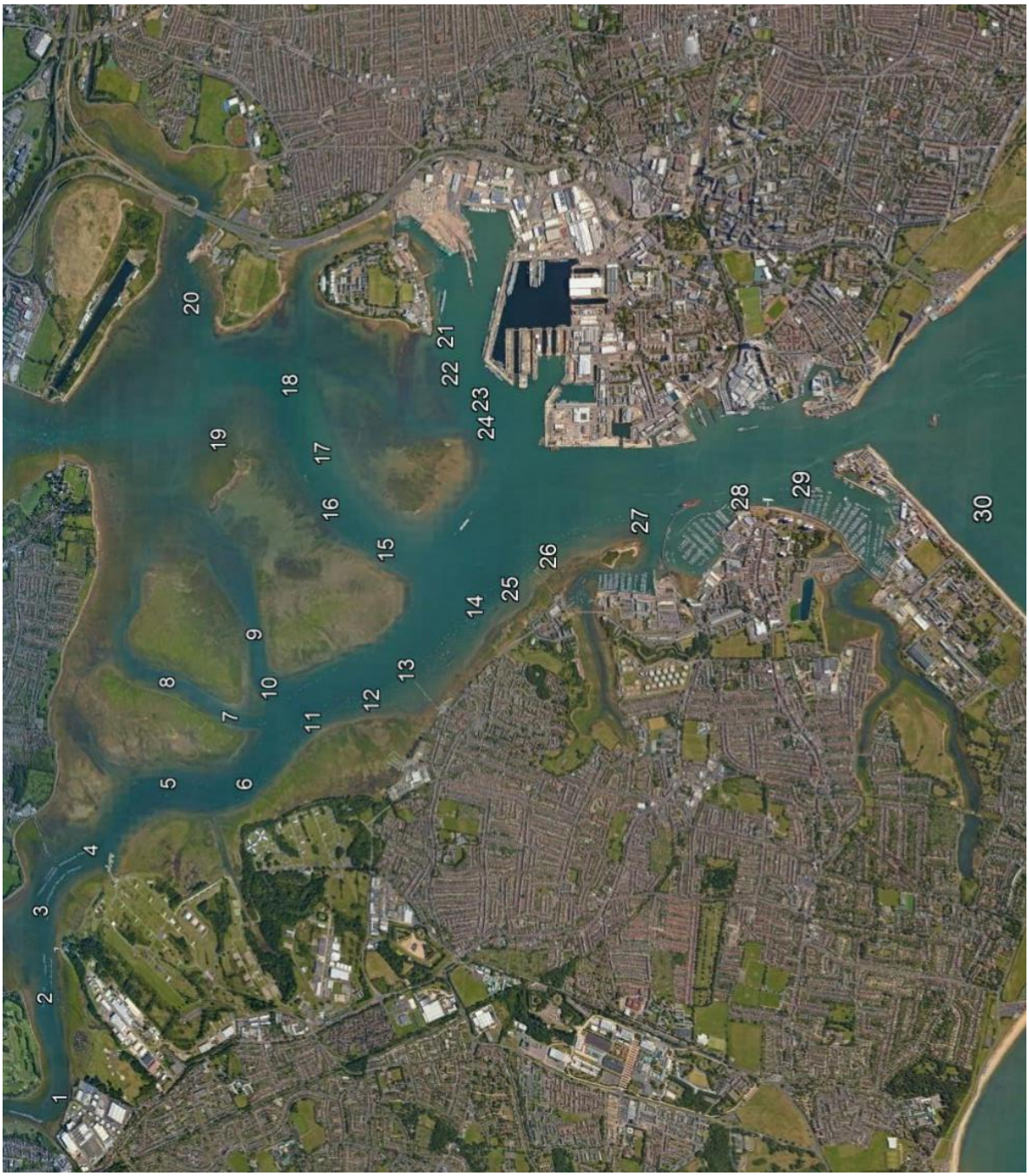
Appendix A

Sample Coordinates for Grab Samples with Corresponding Map Locations

Sampling points within Portsmouth Harbour denoting the location of grab deployment.

Harbour	Sample Site	Latitude (Degrees, decimal minutes)	Longitude (Degrees, decimal minutes)
Portsmouth	1	50 50.324	1 10.365
Portsmouth	2	50 50.385	1 09.801
Portsmouth	3	50 50.399	1 09.305
Portsmouth	4	50 50.179	1 08.930
Portsmouth	5	50 49.851	1 08.527
Portsmouth	6	50 49.543	1 08.504
Portsmouth	7	50 49.596	1 08.139
Portsmouth	8	50 49.851	1 07.965
Portsmouth	9	50 49.498	1 07.686
Portsmouth	10	50 49.441	1 07.979
Portsmouth	11	50 49.274	1 08.149
Portsmouth	12	50 49.050	1 08.021
Portsmouth	13	50 48.919	1 07.857
Portsmouth	14	50 48.668	1 07.515
Portsmouth	15	50 48.990	1 07.224
Portsmouth	16	50 49.196	1 07.010
Portsmouth	17	50 49.225	1 06.707
Portsmouth	18	50 49.347	1 06.338
Portsmouth	19	50 49.632	1 06.623
Portsmouth	20	50 49.735	1 05.863
Portsmouth	21	50 48.812	1 06.165
Portsmouth	22	50 48.748	1 06.302
Portsmouth	23	50 48.689	1 06.503
Portsmouth	24	50 48.621	1 06.854
Portsmouth	25	50 48.543	1 07.418
Portsmouth	26	50 48.409	1 07.249
Portsmouth	27	50 48.097	1 07.068
Portsmouth	28	50 47.772	1 06.954
Portsmouth	29	50 47.579	1 06.894
Portsmouth	30	50 47.039	1 07.015

Corresponding sampling points within Portsmouth Harbour denoting location of grab deployment.



Sampling points within Langstone Harbour denoting the location of grab deployment.

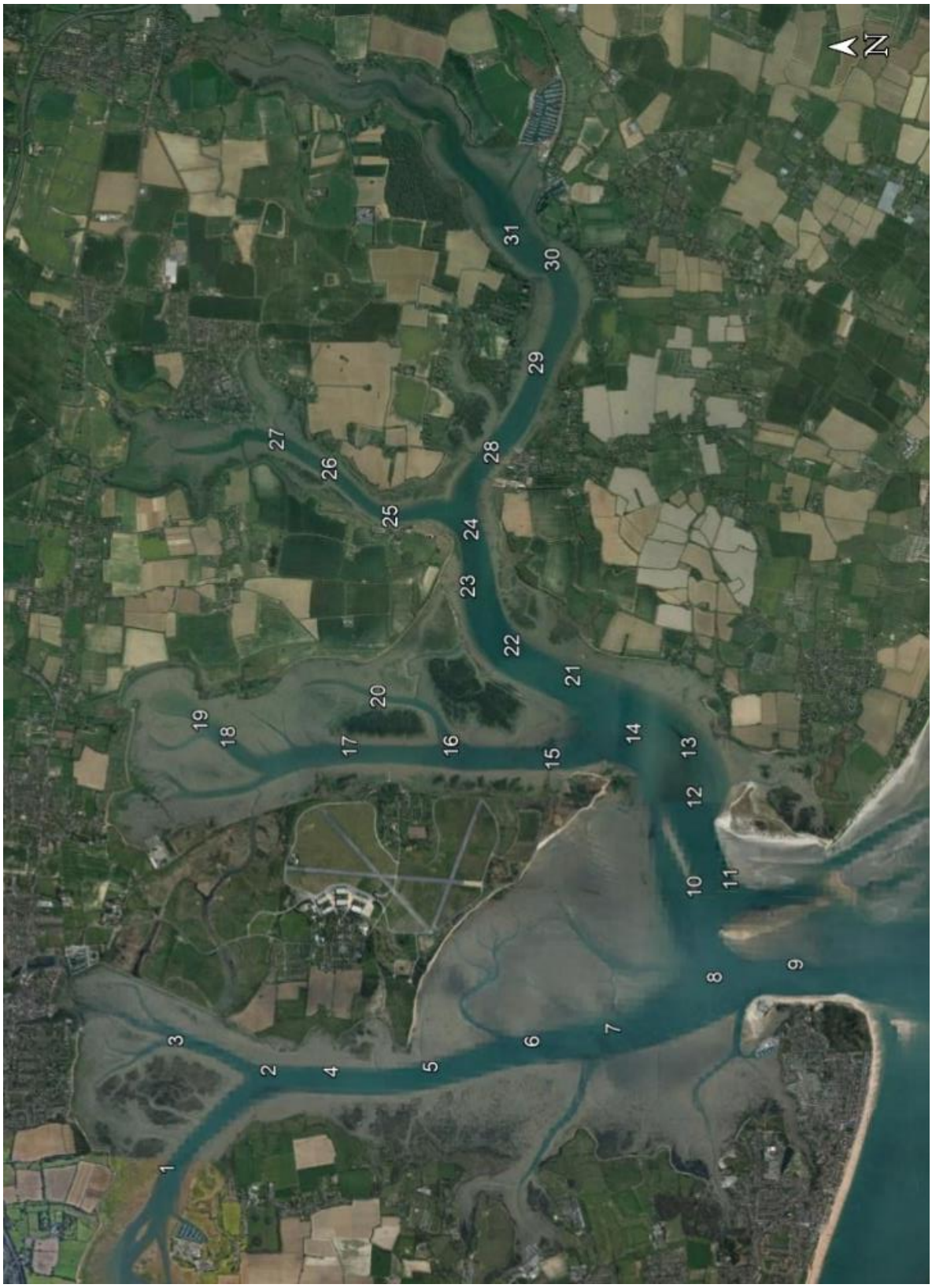
Harbour	Sample Site	Latitude (Degrees, decimal minutes)	Longitude (Degrees, decimal minutes)
Langstone	1	50 49.538	1 02.451
Langstone	2	50 49.276	1 02.250
Langstone	3	50 49.118	1 02.139
Langstone	4	50 48.791	1 01.883
Langstone	5	50 49.264	1 01.298
Langstone	6	50 49.029	1 01.310
Langstone	7	50 48.794	1 01.492
Langstone	8	50 48.604	1 01.764
Langstone	9	50 48.415	1 01.801
Langstone	10	50 48.417	1 01.393
Langstone	11	50 48.324	1 01.642
Langstone	12	50 48.125	1 01.792
Langstone	13	50 48.171	1 01.405
Langstone	14	50 48.055	1 01.517
Langstone	15	50 47.903	1 01.838
Langstone	16	50 47.813	1 01.355
Langstone	17	50 47.939	1 01.185
Langstone	18	50 48.040	1 01.027
Langstone	19	50 48.010	1 0.765
Langstone	20	50 48.007	1 0.562
Langstone	21	50 48.090	1 0.340
Langstone	22	50 48.223	1 0.150
Langstone	23	50 48.240	0 59.997
Langstone	24	50 48.445	1 0.860
Langstone	25	50 48.701	1 00.575
Langstone	26	50 48.707	1 0.172
Langstone	27	50 49.031	1 0.205
Langstone	28	50 49.269	0 59.856
Langstone	29	50 49.477	0 59.838
Langstone	30	50 49.684	0 59.850
Langstone	31	50 49.861	0 59.687

Corresponding sampling points within Langstone Harbour denoting location of grab deployment

Sampling points within Chichester Harbour denoting the location of grab deployment.

Harbour	Sample Site	Latitude (Degrees, decimal minutes)	Longitude (Degrees, decimal minutes)
Chichester	1	50 50.100	0 57.500
Chichester	2	50 49.600	0 56.728
Chichester	3	50 50.056	0 56.497
Chichester	4	50 49.291	0 56.735
Chichester	5	50 48.800	0 56.700
Chichester	6	50 48.300	0 56.500
Chichester	7	50 47.900	0 56.400
Chichester	8	50 47.400	0 56.000
Chichester	9	50 47.000	0 55.900
Chichester	10	50 47.500	0 55.298
Chichester	11	50 47.323	0 55.233
Chichester	12	50 47.500	0 54.600
Chichester	13	50 47.527	0 54.213
Chichester	14	50 47.800	0 54.100
Chichester	15	50 48.200	0 54.300
Chichester	16	50 48.700	0 54.200
Chichester	17	50 49.200	0 54.200
Chichester	18	50 49.800	0 54.129
Chichester	19	50 49.936	0 54.000
Chichester	20	50 49.060	0 53.807
Chichester	21	50 48.100	0 53.640
Chichester	22	50 48.40	0 53.410
Chichester	23	50 48.617	0 52.947
Chichester	24	50 48.600	0 52.500
Chichester	25	50 49.000	0 52.400
Chichester	26	50 49.300	0 52.030
Chichester	27	50 49.559	0 51.795
Chichester	28	50 48.500	0 51.900
Chichester	29	50 48.280	0 51.203
Chichester	30	50 48.200	0 50.400
Chichester	31	50 48.400	0 50.200

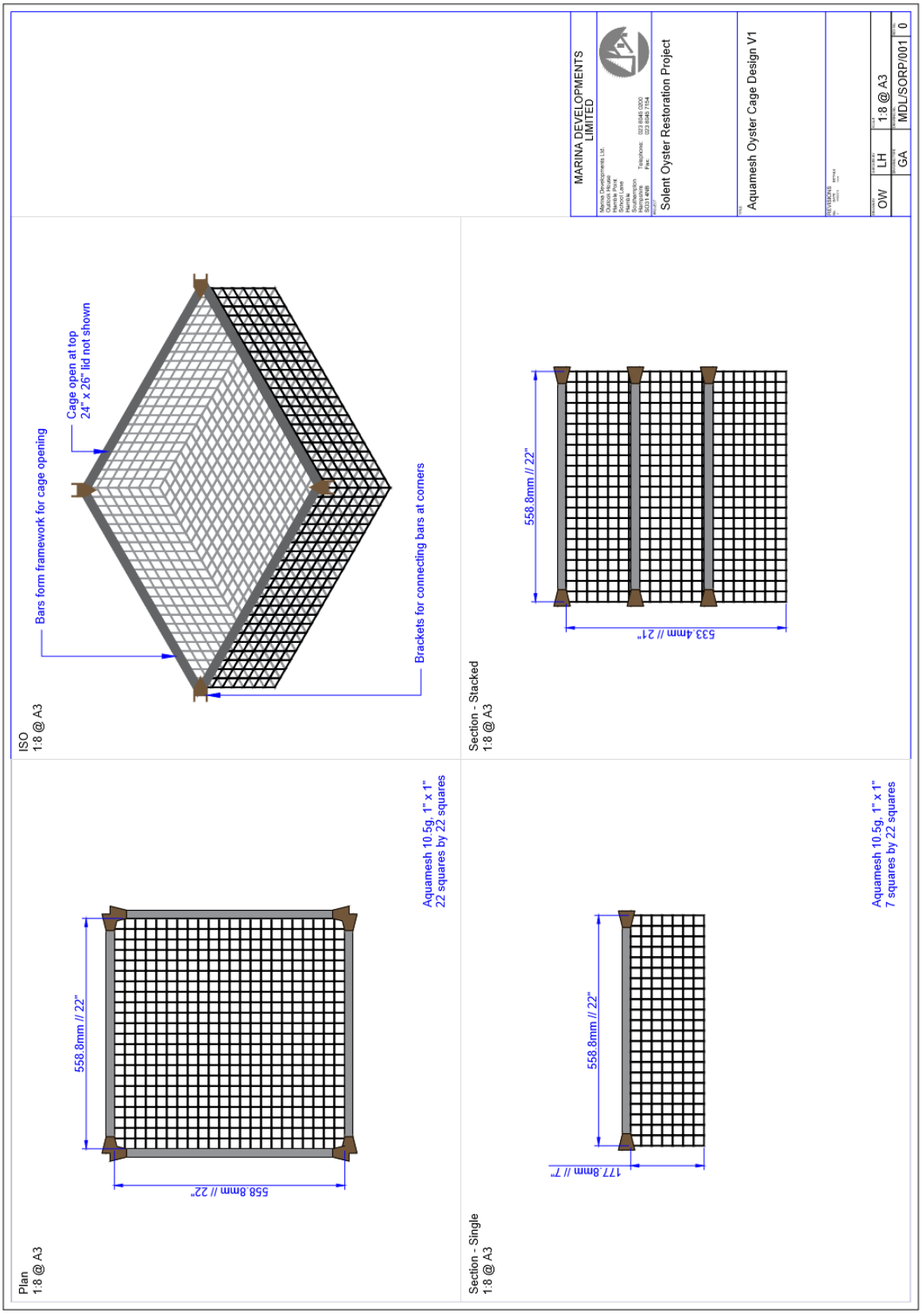
Corresponding sampling points within Chichester Harbour denoting location of grab deployment.



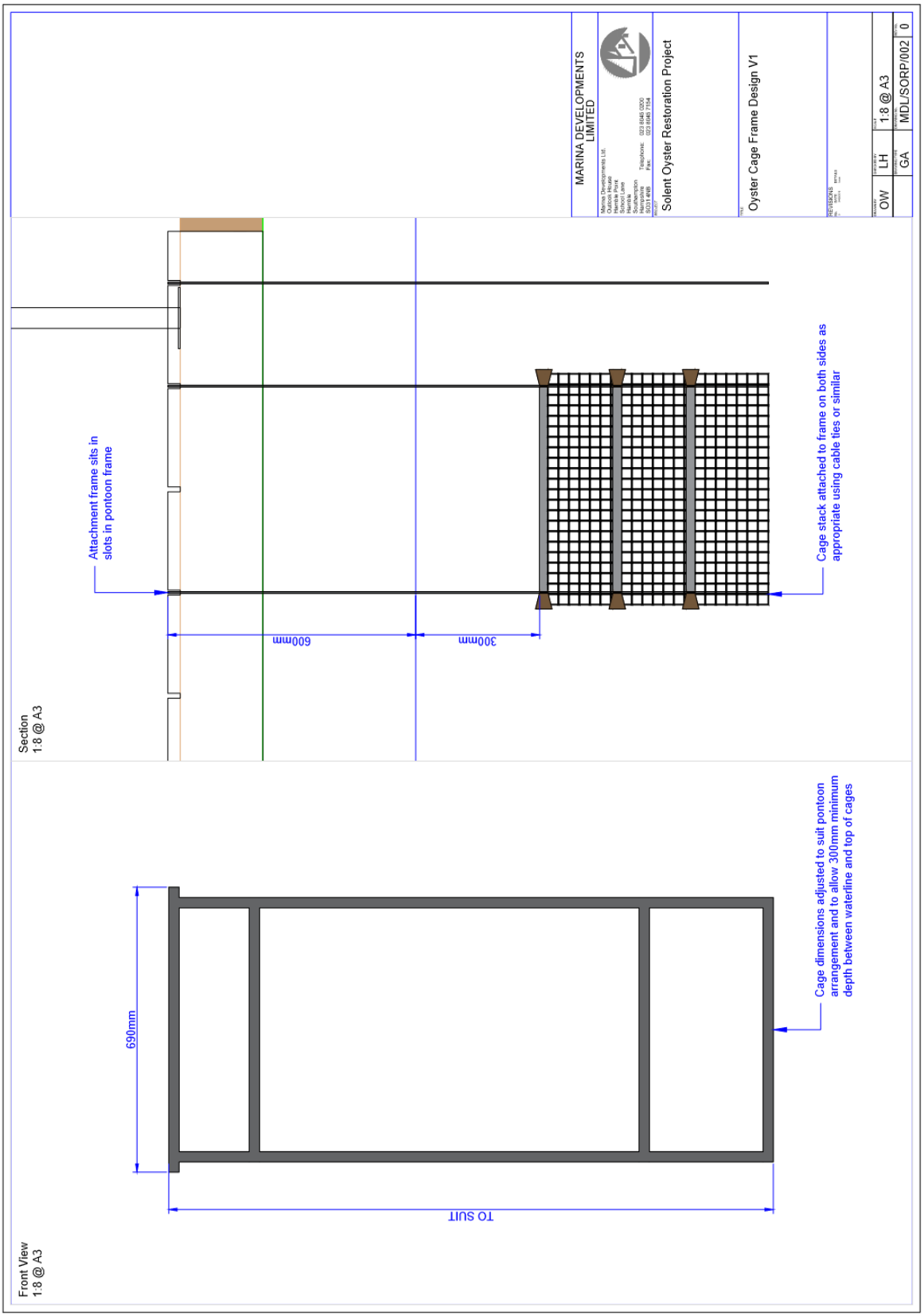
Appendix B

Broodstock Cage Design Blueprints, 2015 - 2016

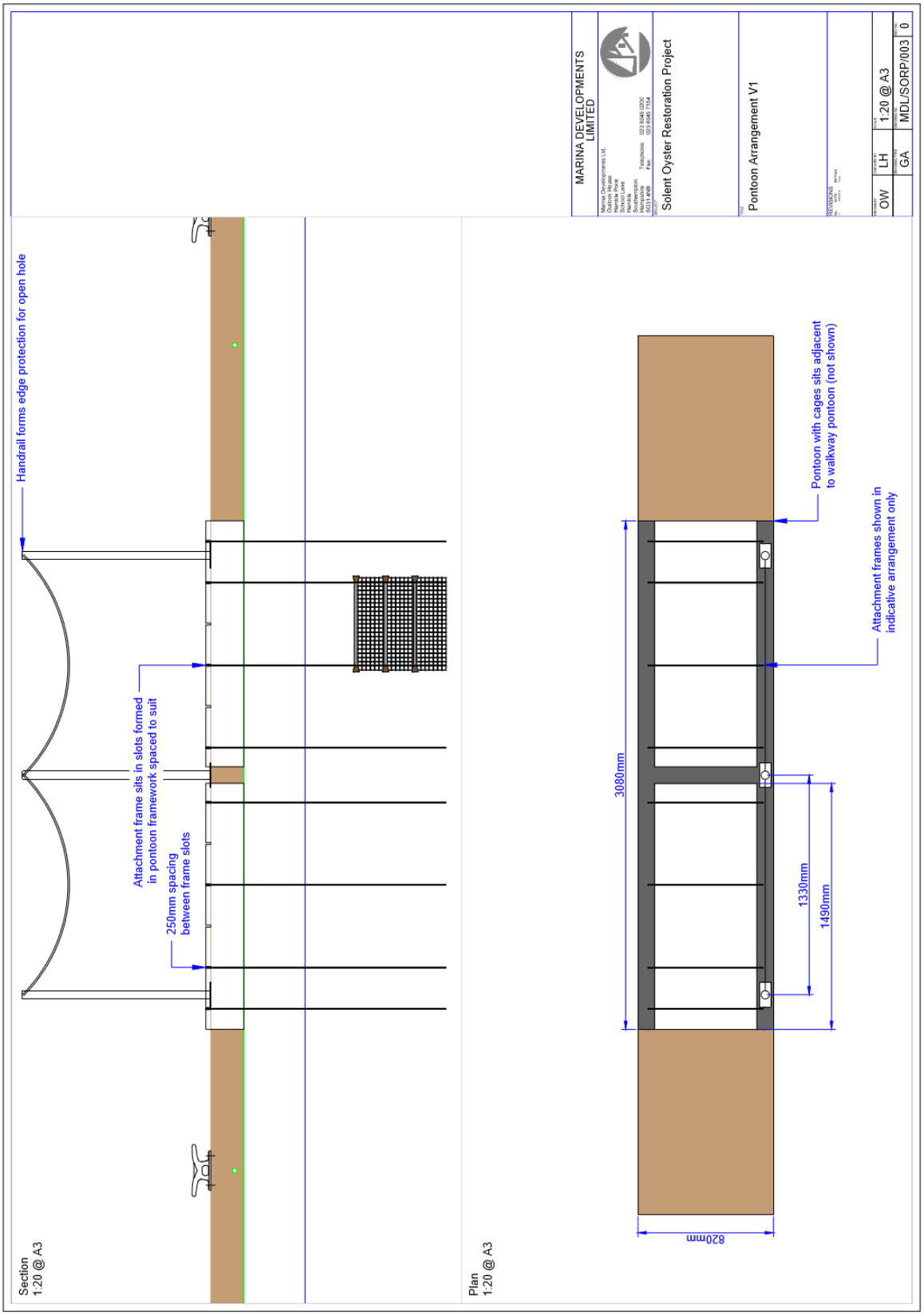
Broodstock cage design showing assembly of individual cage units into a stack structure.



Attachment mechanism used to suspend broodstock cages in the marina environment.



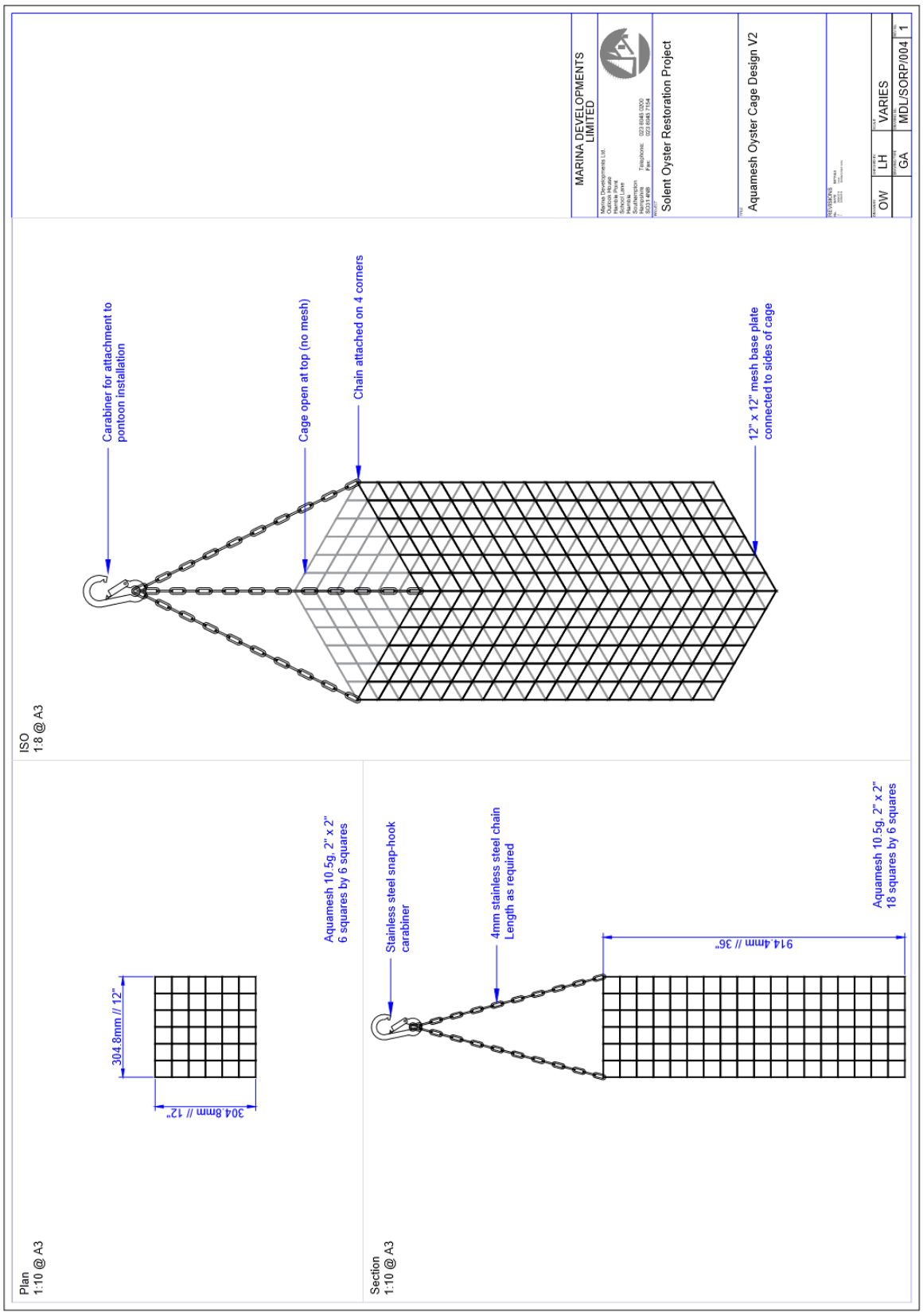
Pontoon structure and modifications to house broodstock units on pontoon structures.



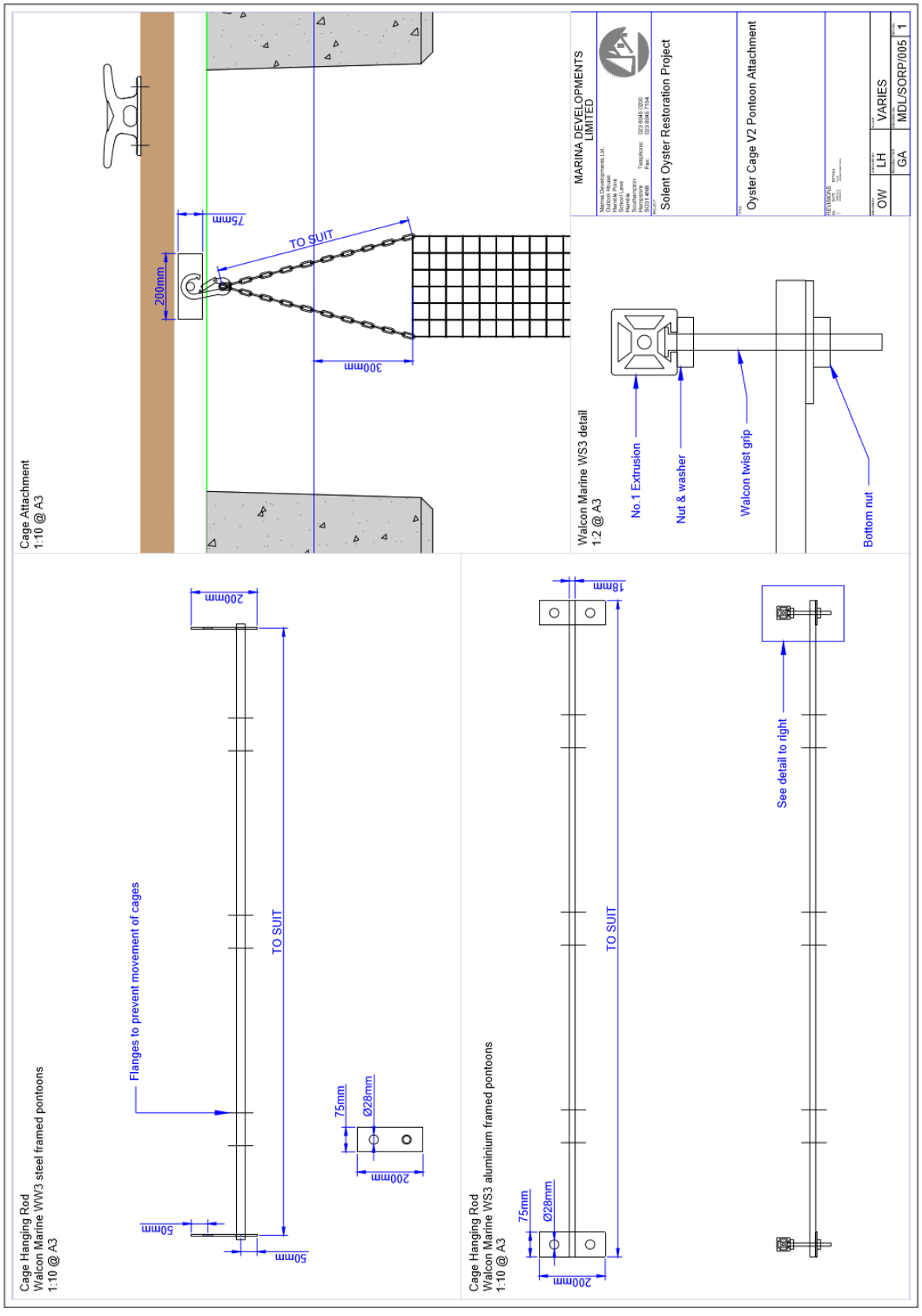
Appendix C

Broodstock Cage Design Blueprints, 2017 - 2018

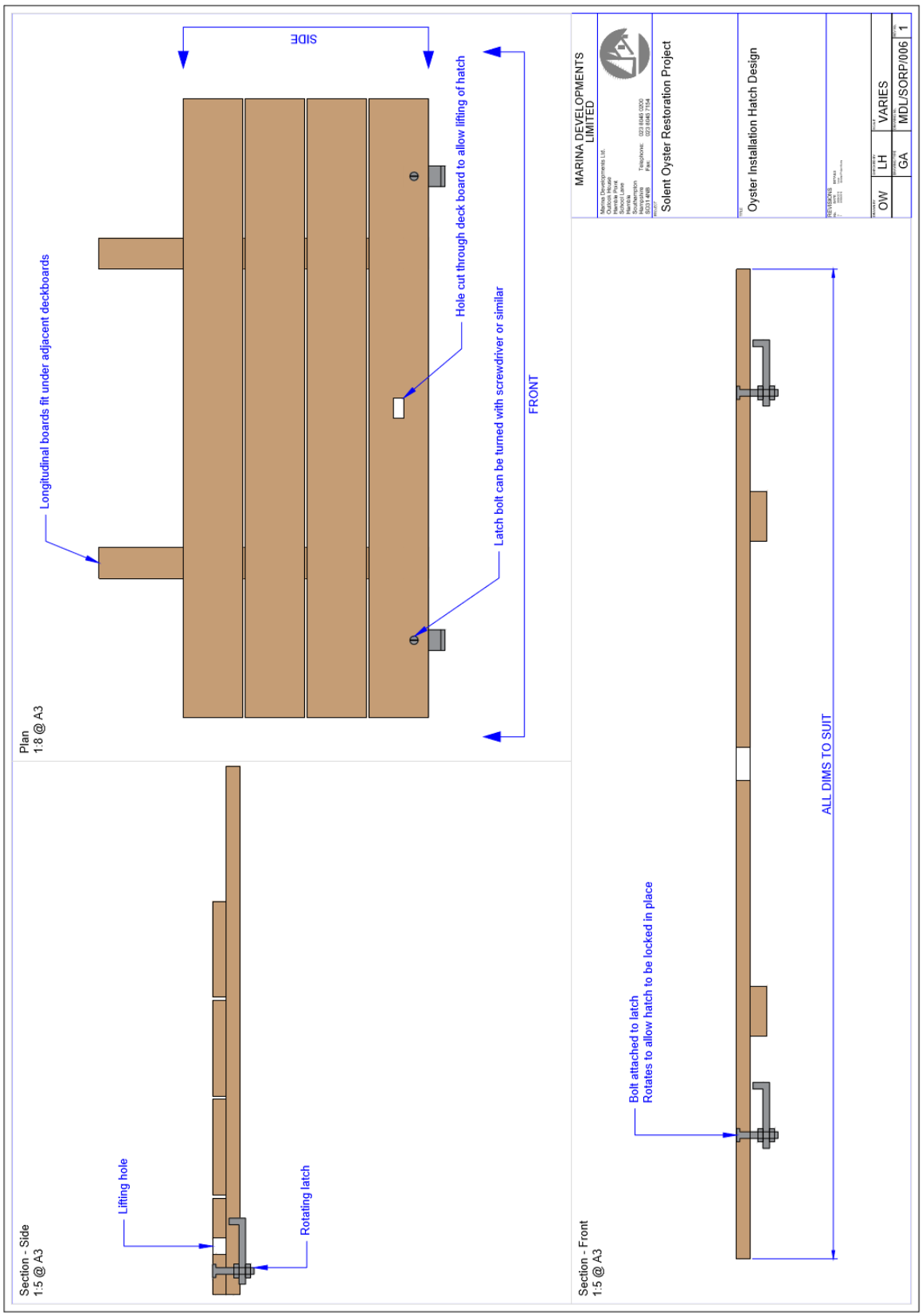
Suspended broodstock cage design.



Pontoon modifications made to enable suspension of broodstock cages.



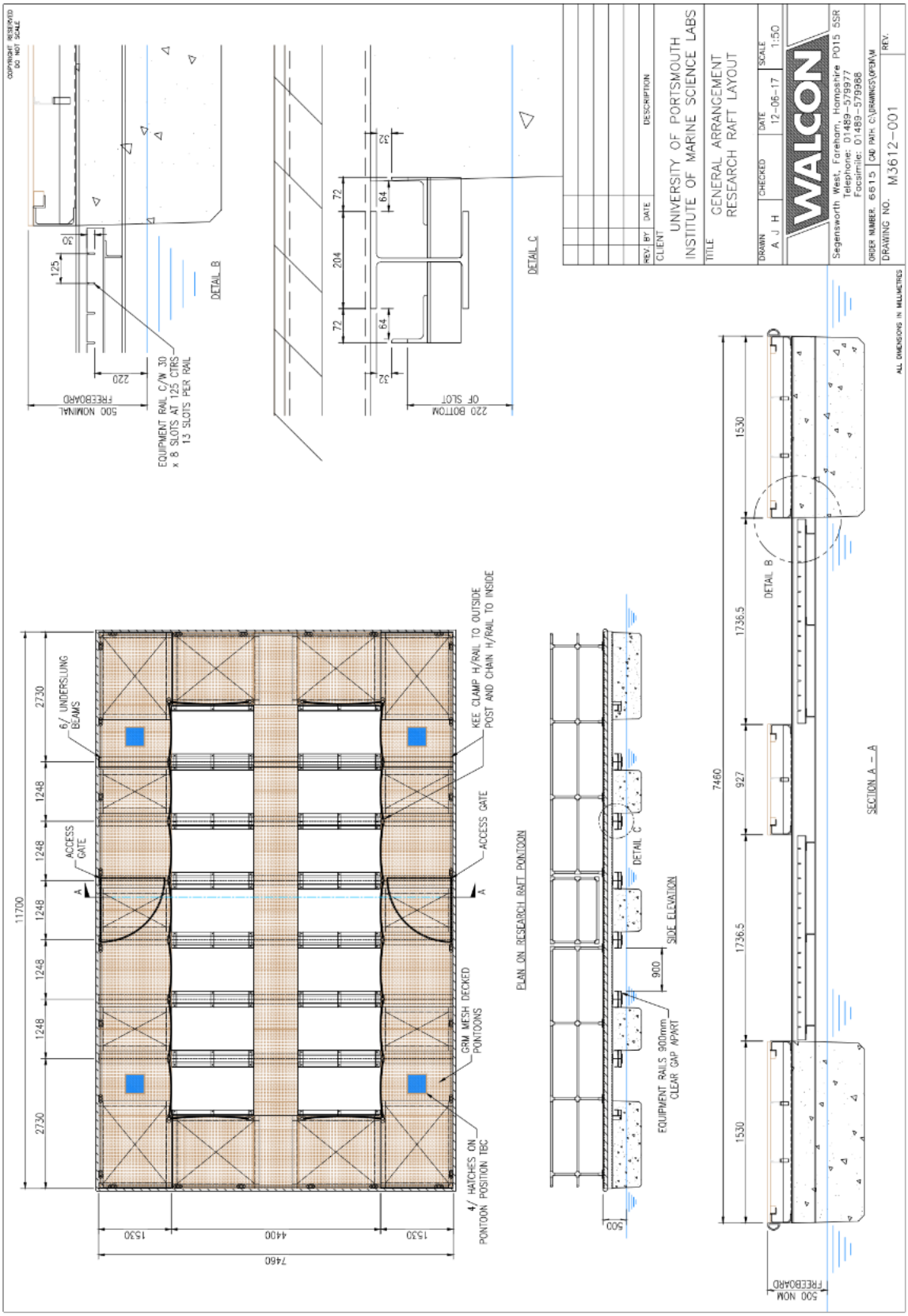
Pontoon modifications made to allow access to suspended broodstock cages.



Appendix D

University of Portsmouth Research Platform Blueprints

University of Portsmouth raft design and dimensions.



Appendix E

Reference Samples Selected for Purification and Sequencing for Disease Prevalence

List of samples obtained that were sequenced for species identification and conformation. See Figure 3.X for location reference.

Sample group	Primer set	Location	Extraction number	Positive ID
2016 Broodstock	Oe	BA	2	Y
2016 Broodstock	BO/BOAS	UP	47	Y
2016 Broodstock	BOSTRE	BA	16	Y
2016 Broodstock	BOSTRE	UP	43	Y
2016 Broodstock	BEXIT	BA	24	N
2016 Broodstock	BEXIT	UP	54	N
2016 Broodstock	C F/R	UP	47	Y
2016 Broodstock	C F/R	UP	53	Y
2017 Brooding Adults	Oe	UP	1	Y
2017 Brooding Adults	BO/BOAS	BA	6	Y
2017 Brooding Adults	BOSTRE	BA	6	Y
2017 Brooding Adults	C F/R	BA	6	Y
2017 Broodstock	Oe	SW	1	Y
2017 Broodstock	BO/BOAS	SW	1	Y
2017 Broodstock	BOSTRE	SW	1	Y
2017 Broodstock	BEXIT	PH	31	Y
2017 Broodstock	BEXIT	PH	34	Y
2017 Broodstock	BEXIT	HP	50	N

Sample group	Primer set	Location	Extraction number	Positive ID
2017 Broodstock	BEXIT	BA	65	Y
2017 Broodstock	C F/R	SW	12	Y
2017 Broodstock	C F/R	SW	13	N
2017 Broodstock	C F/R	PH	33	N
2017 Broodstock	Folmer	SP	95	Y
2017 Broodstock	BO/BOAS	SP	95	Y
2017 Broodstock	BOSTRE	SP	95	Y
2017 Spat	Oe	BA	100	Y
2017 Spat	BO/BOAS	BA	100	Y
2017 Spat	BOSTRE	BA	100	Y
2018 Broodstock	Oe	PH	23	Y
2018 Broodstock	BO/BOAS	PH	24	Y
2018 Broodstock	BOSTRE	PH	23	Y
2018 Broodstock	BOSTRE	PH	24	Y
2018 Broodstock	BOSTRE	UP	68	Y
2018 Broodstock	BEXIT	PH	23	N
2018 Broodstock	BEXIT	HP	35	N
2018 Broodstock	BEXIT	BA	56	N
2018 Broodstock	BEXIT	UP	68	N
2018 Broodstock	C F/R	PH	23	N

Sample group	Primer set	Location	Extraction number	Positive ID
2018 Broodstock	C F/R	UP	62	Y
2015 Seabed	Oe	H+S	P2	Y
2015 Seabed	BO/BOAS	H+S	P2	N
2015 Seabed	BEXIT	H+S	P20	Y
2015 Seabed	Oe	E/T	C5	Y
2015 Seabed	BO/BOAS	E/T	C5	Y
2015 Seabed	C F/R	E/T	C5	Y
2015 Seabed	BEXIT	E/T	C20	N
2015 Seabed	BEXIT	E/T	C24	N
2015 Seabed	BEXIT	E/T	C39	N
2017 Larvae	Oe	UP	3	Y
2017 Larvae	BO/BOAS	UP	3	Y
2017 Larvae	BOSTRE	UP	3	Y
2017 Larvae	BEXIT	UP	3	N
2017 Larvae	BEXIT	PH	8	Possible
2017 Larvae	BEXIT	SP	13	Y
2018 Larvae	BOSTRE	SP	14	Y
2018 Larvae	BEXIT	SP	14	Y
2017 Larvae	BEXIT	SP	18	Possible
2017 Larvae	BEXIT	SP	24	Possible
2017 Larvae	BEXIT	SP	26	Possible
2017 Larvae	BEXIT	UP	30	Possible
2017 Larvae	BEXIT	HP	31	N

Appendix F

University of Portsmouth UPR16 Form

FORM UPR16

Research Ethics Review Checklist

Please include this completed form as an appendix to your thesis (see the Research Degrees Operational Handbook for more information

UNIVERSITY OF PORTSMOUTH

Postgraduate Research Student (PGRS) Information			**Student ID:**	UP609247
PGRS Name:	Luke David Helmer			
Department:	School of Biological Sciences	**First Supervisor:**	Dr Joanne Preston	
Start Date: (or progression date for Prof Doc students)	**1/10/2016**			
Study Mode and Route:	Part-time ☐ Full-time ☒	MPhil ☐ PhD ☒	MD ☐ Professional Doctorate ☐	

Title of Thesis:	The efficacy of suspended broodstock cages as a restoration strategy for the European flat oyster Ostrea edulis Linnaeus, 1758: A case study in the Solent, UK.
Thesis Word Count: (excluding ancillary data)	47,689

If you are unsure about any of the following, please contact the local representative on your Faculty Ethics Committee for advice. Please note that it is your responsibility to follow the University's Ethics Policy and any relevant University, academic or professional guidelines in the conduct of your study

Although the Ethics Committee may have given your study a favourable opinion, the final responsibility for the ethical conduct of this work lies with the researcher(s).

UKRIO Finished Research Checklist:
(If you would like to know more about the checklist, please see your Faculty or Departmental Ethics Committee rep or see the online version of the full checklist at: http://www.ukrio.org/what-we-do/code-of-practice-for-research/)

a) Have all of your research and findings been reported accurately, honestly and within a reasonable time frame?	YES ☒ NO ☐
b) Have all contributions to knowledge been acknowledged?	YES ☒ NO ☐
c) Have you complied with all agreements relating to intellectual property, publication and authorship?	YES ☒ NO ☐
d) Has your research data been retained in a secure and accessible form and will it remain so for the required duration?	YES ☒ NO ☐
e) Does your research comply with all legal, ethical, and contractual requirements?	YES ☒ NO ☐

Candidate Statement:	
I have considered the ethical dimensions of the above named research project, and have successfully obtained the necessary ethical approval(s)	
Ethical review number(s) from Faculty Ethics Committee (or from NRES/SCREC):	919A
If you have *not* submitted your work for ethical review, and/or you have answered 'No' to one or more of questions a) to e), please explain below why this is so:	
Signed *(PGRS)*:	**Date:** 30/09/2019

UPR16 – April 2018

Appendix G

University of Portsmouth Ethics Form

26th September 2019

University of Portsmouth

Professor Matt Guille
School of Biological Sciences
King Henry Building
King Henry I Street
Portsmouth PO1 2DY
England

Tel: +44 (0)23 9284 2047
Fax: +44 (0)23 9284 2070
email: matthew.guile@port.ac.uk

Dear Dr Preston,

RE: Ethics submission – **The efficacy of suspended broodstock cages as a resotration strategy for the European flat oyster *Ostrea edulis* Linnaeus, 1758: A case study in the Solent, UK.**

Approval of project by the Animal Welfare and Ethical Review Body (AWERB)

I am very happy to confirm that we were able to fast track your application and that the AWERB gave its approval for your proposal concerning work within the above project.

The AWERB uses UK Home Office guidelines on the Animals (Scientific Procedures) Act 1986 when assessing proposals and adheres to the regulations of the European Directive 2010/63/EU. Your project has been assessed as not falling within A(SP)A because it does not involve a protected species. We are confident that the proposal demonstrates appropriate consideration of the Three Rs and animal welfare. Please use this letter as confirmation of ethical approval from AWERB, University of Portsmouth. Please use the number 919A as confirmation of the successful review.

Yours sincerely,

MJ Guille PhD FSB
Professor of Developmental Genetics and Chair, AWERB